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Exploring Mechanisms of Adaptive Divergence in an Exemplar Adaptive Radiation

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Abstract

Investigating and exploring mechanisms of adaptive divergence is key to understanding how complex morphological traits have evolved. Exemplar systems of adaptive radiation, whereby numerous species have diverged from a common ancestor in a relatively rapid timeframe, can be used to test ideas about adaptive divergence. Adaptations of the trophic morphology are often the focus of divergence in adaptive radiations, but the evolution of such traits is not yet fully understood. With extensive variation in craniofacial shape, the Lake Malawi cichlids are an excellent system which can be used to investigate the evolution of trophic morphology. Traditional studies of divergence tend to focus on the relationship between shape and ecology, but an evo-devo approach which encompasses multiple aspects such as morphology, genetics, function and development can address questions about the evolutionary process in more detail. Furthermore, investigations which look at smaller scale patterns of divergence, such as between ecologically similar species or between sexes, can be especially enlightening as this can uncover more subtle aspects of variation. Ecological sexual dimorphism, whereby sexes diverge in ecologically relevant traits such as the trophic morphology, can represent one such type of subtle variation. This thesis explores the evo-devo of the trophic morphology with an interdisciplinary approach by considering multiple levels of adaptive divergence and their contribution to evolutionary process. **Chapter 1** sets out of the context of these investigations and the background for this work.

Firstly, the genetic basis of the mandible is explored in **Chapter 2** to uncover new candidate genes. The mandible represents the first point of contact with the environment and as such is a key vertebrate trait, yet the complexity means the underlying genetic architecture is not fully understood. By investigating the genotype to phenotype relationship in high detail, I found a candidate gene not previously characterised in cichlid craniofacial studies, *zeb1*. Furthermore, there was strong evidence of sexual dimorphism in mandible shape and mapping highlighted regions for quantitative trait loci on the sex-determining chromosome. Following on from this, **Chapter 3** utilised an engineering technique, finite element analysis, to assess how the mandible copes with

external compressive loading that would be expected during feeding. This analysis identified key structural adaptations in both species to enable them to cope with stress during feeding, and notably there was strong dimorphism between sexes. The final experimental chapter, **Chapter 4**, assessed phenotypic plasticity through a diet treatment experiment with the main aim to investigate sexual dimorphism in plastic response. Despite strong sexual dimorphism in morphology and function, plastic responses did not differ between the sexes. This is in spite of the fact that females are mouthbrooders, but this does not appear to place constraints on phenotypic plasticity. Discussed in detail in **Chapter 5**, the work presented in this thesis suggests that adaptive divergence between species and sexes could both be important to the evolution of the Lake Malawi radiation. By using an integrative approach which considers multiple mechanisms of divergence, this can enhance our understanding of the evolution of complex traits and the evolutionary process itself.

"All we have to decide is what to do
with the time that is given us."

J.R.R TOLKIEN

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
bmp4	Bone morphogenetic protein 4
cam1	Calmodulin
col1a1	Collagen 1
DIG	Digoxigenin
EES	Extended Evolutionary Synthesis
ESD	Ecological Sexual Dimorphism
FEA	Finite Element Analysis
Fst	Fixation Index
IOP	Interopercle
JP	Jaw protrusion
LF	*Labeotropheus fuelleborni*
LG	Linkage group
LOD	Logarithm of the odds
MABT	Maleic acid buffer
MAo	Mechanical advantage of opening
MQM	Multiple QTL mapping
MZ	*Maylandia zebra*
NCC	Neural crest cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PCA	Principle components analysis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PPD	Partial Procrustes distance
QTL	Quantitative trait loci
RA	Retroarticular
RAD-seq	Restriction site-associated DNA sequencing
SSC	Sodium citrate solution
SNP	Single Nucleotide Polymorphism
TRC	*Tropheops* “Red Cheek”
μ-CT	Micro Computerised Tomography
WISH	Whole mount *in situ* hybridisation
wnt	Wingless
zeb1	Zinc finger homeobox 1

List of Collaborations

Chapter 2

I collected the mandibles from Dr R. Craig Albertson, University of Massachusetts, Amherst. I scanned the mandibles at the University of Strathclyde with Dr Jeremy Gibson in the laboratory of Dr James Windmill. The shape work was conducted in collaboration with Professor Adrian Bowman, Dr Liberty Vittert and Yinuo Liu, at the University of Glasgow; they created the curves using the shape index and assisted with generating the morphometric data.

Chapter 3

I collected the mandibles from Dr R. Craig Albertson, University of Massachusetts Amherst. I scanned the mandibles at the University of Strathclyde with Dr Jeremy Gibson in the laboratory of Dr James Windmill. I conducted finite element analysis with guidance from Professor K. Elizabeth Tanner and Dr Magnus Kjartan Gislason.

Chapter 4

Bethany Smith photographed the fish for the first set of craniofacial landmarks.

Appendix 1

I contributed this section to the book chapter “An evo-devo view of post-genomic African cichlid biology: enhanced models for evolution and biomedicine” with Dr Kevin Parsons and Tiffany Armstrong which has been submitted for publication.

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Author's Declaration

The research conducted in this thesis took place between October 2015 and September 2019 and is entirely my own, unless otherwise stated in the List of Collaborations. None of the work in this thesis has been submitted for another degree.

Kirsty McWhinnie
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Chapter 1: General Introduction

1.1 Adaptive Phenotypic Divergence

Adaptive divergence involves the evolution of species or populations adapted to different ecological niches from a common ancestor. Related to this, adaptive radiations result in the evolution of multiple species within a lineage over a rapid time frame (Schluter, 2000). Notable examples of adaptive divergence include three-spine sticklebacks (*Gasterosteus aculeatus*) in British Columbia where there are six instances of sympatric species pairs that are morphologically differentiated to specialise in benthic and limnetic habitats and feed on different types of prey (Rundle, Vamosi and Schluter, 2003). In these pairs, the limnetic specialist, which generally feeds on plankton, has a narrower mouth and more, longer gill rakers and is generally smaller in size than the benthic specialists, which tend to feed on larger invertebrates (Schluter and McPhail, 1993; Schluter, 1996; Bolnick and Lau, 2008). Similar phenotypic divergence along a benthic/limnetic axis has taken place in Arctic charr (*Salvelinus alpinus*) in postglacial lakes in Iceland and Scotland (e.g. Snorrason *et al*. 1994; Adams *et al*. 1998). While such adaptive divergence has been the focus of a large amount of empirical research, it is important to recognise adaptive changes can also occur at other levels of biological organisation; sexes can adaptively diverge to suit different niches, a phenomenon known as ecological sexual dimorphism (hereafter ESD). However, ESD has rarely been investigated making it unknown how widely this phenomenon may contribute to processes of adaptive divergence and radiation (Shine, 1989; Cooper, Gilman and Boughman, 2011).

Adaptive divergence in populations may represent an early step of the three-stage model proposed to explain the process of species formation via adaptive radiation (Streelman and Danley, 2003). In this model the first stage involves divergence based on habitat, such as in the stickleback example above where divergence occurs between benthic and limnetic habitats (Rundle, 2002; Streelman and Danley, 2003). The next stage of the model involves divergence in trophic morphology such as in the numerous trophic adaptations in African

cichlids including piscivores, planktivores, insectivores and molluscivores (Fryer and Iles 1972; Albertson *et al*. 1999; Streelman and Danley, 2003). The final stage of the model involves a diversification of phenotypes for communication. African cichlids are notable examples of this, whereby differences in male mating colouration is prevalent and proposed to be as a result of sexual selection (Deutsch, 1997; Streelman and Danley, 2003; Kocher, 2004). Whilst this model represents a general example of how adaptive divergence can proceed toward speciation, it is important that fine-scale, ecologically salient variation at different levels be considered as contributors to the process of adaptive radiation as a whole (Parsons *et al*. 2015 and references therein).

Disruptive selection, usually thought to be due to ecological differences between habitats, can drive evolutionary divergence that reduces competition between individuals (Cooper, Gilman and Boughman, 2011). Sexual dimorphism can evolve because of differences in reproductive effort between sexes or due to differences in ecology. However, ESD, as a type of adaptive divergence, can result in a reduction of competition between sexes. As ESD can also be the product of the same ecological circumstances as adaptive speciation, and whichever of the two forms evolves first may reduce the disruptive selection needed for the other to evolve, this has led to the suggestion that both processes are "two sides of the same ecological coin" meaning that the two would not be expected to co-occur in the same population (Bolnick and Doebeli 2003; Cooper, Gilman and Boughman, 2011). Positive assortative mating is thought to be incompatible with ESD because females cannot choose both a male ecologically similar to herself while still maintaining sexual dimorphism within the species (Bolnick and Doebeli, 2003). However, ecological modelling has predicted that speciation may be compatible with ESD if the traits that the mates prefer do not have an ecological function (Bolnick and Doebeli, 2003). Therefore, ESD has probably been neglected due to concerns that it cannot co-exist with speciation.

Previous studies of adaptive divergence have often focused on "form to ecology" relationships. For example, beak shape in Darwin's finches and how it corresponds to diet which in turn informs which environments are inhabited was

first reported by Lack (1947); these ideas formed the basis of research on the adaptive radiation. Establishing the link between form and ecology then led to functional work in this group; research on the bite force, and therefore crushing ability, of these finches found a correlation between multiple measures of beak shape with biting force and this brought about multiple studies assessing various aspects of the relationship between function, form and ecology in more detail (Herrel *et al*. 2005, 2009; Soons *et al*. 2010). Functional studies can offer ideas on why morphological change occurs. To gain a further understanding of the link between morphology, function and ecology, Wainwright (1994) suggests the use of performance testing. According to Wainwright (1994), an organism's performance is its ability to behave and carry out tasks, with the phenotype of the organism determining the boundaries of performance. These boundaries determine the resources that individuals can utilise and result in fitness consequences. For example, performance testing of feeding specialisations can involve comparing species with different trophic morphologies and diets and assessing how successful they are when their diets are reciprocally switched (e.g. Bouton, Van Os and Witte, 1998). This provides a means for determining how phenotypes react with different environments or resources.

Performance testing has been used extensively in *Anolis* lizards (e.g. Losos and Sinervo, 1989; Losos, Warheitt and Schoener, 1997; Losos, 1998) to produce predictions about how certain morphological features function and have an effect on an organism's performance in its environment (Wainwright, 1994). The *Anolis* lizard radiation in the Caribbean Islands provide an excellent example of how functional morphology studies can be carried out in conjunction with research on adaptive divergence (Losos, 1990; Losos and Irschick, 1996). Each island possesses a similar set of ecomorphs which have evolved independently and are divergent in morphology, ecology and behaviour (Losos, 1998). They all differ in fore-limb, hind-limb and tail length, as well as body shape and size; all of these features impact on how they perform in the environment (Losos, Irschick, and Schoener, 1994). The limb length and body size effects how the animal can perform on different surfaces in its environment; studies have shown how the function of having long legs and heavy bodies in *Anolis* lizards is to perform better on wide perches and jump farther than those with shorter legs

(Losos, 1990). The relationship between function, form and ecology is now well understood in the *Anolis* lizard radiation and this should now be used as a starting point to explore other examples of adaptive radiation and potential cases of ESD.

Adaptations in trophic morphology have been key to many examples of adaptive radiation (e.g. *Geospiza*, Haplochromines). Specifically, the shape and structure of the jaw is subject to bite force limits that determine prey use (Wainwright, 1994). For example, an investigation of feeding ability in species of Caribbean wrasse (*Halichoeres* sp.) showed that the pharyngeal jaw has a functional role through its crushing strength which constrains the dietary choices (Wainwright, 1988). Furthermore, it was suggested that the fish specialised for crushing hard-shelled prey effectively did so at the expense of being competent in feeding on soft bodied prey (Wainwright, 1988). This highlights the important role trophic morphology has in determining diet as well as the potential trade-offs in function that can result from specialisation. Studying the relationship between function, morphology and ecology can form an important part of research into the evolution of morphological specialisation in examples of adaptive divergence.

Similarly, examples of ESD display features consistent with functional change, but explicit investigation from this perspective has been largely unexplored. Functional studies could be vital for determining the prevalence of ESD by determining whether sexual dimorphisms, which are commonly observed, provide functional advantages. Currently, functional morphological studies are increasingly merging with engineering by adopting powerful techniques to understand biomechanical variation. Additionally, how form and function are genetically controlled and develop is becoming an increasingly hot topic as it can provide additional insights into the evolutionary process (Irschick *et al*. 2013). Taken together, an interdisciplinary approach can be especially powerful for discovering the underpinnings of adaptation and raises exciting new questions and opportunities for further study.

1.2 Utilising Biomechanical Modelling to Investigate Function

To comprehensively analyse the relationship between form and function, and how this relates to ecology, biomechanical modelling is an emerging technique that can be used. Indeed, Polly *et al*. (2016) proposed a framework that combines the techniques of morphometrics and biomechanical modelling to answer the evolutionary questions. Using biomechanics as a means of investigating an ecologically important trait can provide greater insight into why adaptation has occurred. For example, biomechanical modelling techniques such as finite element analysis (FEA) can combine three-dimensional (3D) shape of any object with the modelling of force transfer. FEA divides the structure of a three-dimensional model into separate areas called elements which are all joined together at their vertices, referred to as nodes. The model is then turned into a mesh by the FEA to represent the geometry of the shape. The mesh is then assigned both material and structural properties to control how the model will change under stress. By applying forces and constraints relevant to the organism, function can be examined. The stress and strains experienced can then be displayed on the model in different colours to reflect in the magnitude (Panagiotopoulou, 2009). FEA has proven to be a useful tool for analysing relationships in form and function in evolutionary biology where it is applied to a wide variety of biomechanical and functional studies including adaptive divergence (Panagiotopoulou, 2009).

Given that the face interacts directly with prey through foraging, it is not surprising that the craniofacial region is a key component for many examples of adaptive divergence. Therefore, determining the biomechanics of feeding morphology using newer techniques (e.g. FEA) can provide a more comprehensive understanding of adaptation. Techniques such as 3D modelling and FEA represent a major recent improvement over standard methodology (i.e. simple linear measures based on lever mechanics) as they offer a more direct way of testing and visualizing function in terms of force transfer across an entire form. This can enhance traditional measures of function and force inferred from shape and muscle mass to approximate biomechanical abilities. For example,

when Wainwright (1987) estimated the feeding ability of the Caribbean hogfish *Lachnolaimus maximus* (a mollusc crusher), the value for the maximum potential crushing force of the pharyngeal jaw was inferred from calculations of potential force capability performed on muscles associated with biting (Wainwright, 1987). Measuring the muscles associated with the jaw to estimate theoretically maximal bite force could have resulted in an underestimation of the true bite force as this method has a high level of error associated with measurements (Huber, Weggelaar and Motta, 2006).

Research has moved towards incorporating FEA, which although an indirect technique, is much more comprehensive in how it takes account of form. As a technique for analysing the biomechanics of form and function, FEA is advantageous to use because both force and stress, as well the material properties of the bone can be modelled together (Ferrara *et al*. 2011). By including these details, FEA is particularly useful for investigating the biomechanics of natural variation in animals. For example, in Darwin's Finches, FEA modelling by Soons *et al*. (2010) has shown that species with deep and wide beaks are able to dissipate stress better than those with long beaks. In addition, having this beak shape allows for the reduction of areas which would normally be under high stress; they are then able to crack hard seeds with a reduced risk of the beak breaking. In sharks, FEA has been utilised as a means to understand the link between jaw mechanics and feeding; in a comparison between great white (*Carcharodon carcharias*) and sand-tiger (*Carcharias taurus*) sharks, Ferrara *et al*. (2011) used FEA to show that differences in bite force and bite velocity between the two species are related to their diets and dentition. Therefore, FEA proves an important technique to incorporate into studies of the adaptive divergence of function, form and ecology.

While FEA can be advantageous, it also presents some drawbacks in practice. For example, FEA models often do not feature directly derived material properties for the structure under study which compromises accuracy (Korioth and Versluis, 1997). This problem can be eliminated by experimentally testing material properties but is often difficult and rarely conducted. For example, Hulsey *et al*. (2008) used FEA to determine the stress inflicted on the pharyngeal jaw of a

Herichthys minckleyi (a neotropical cichlid) morph when eating hard prey and had to use bone material properties which were described for similar structures in other vertebrates as there were none described for this species. The storage of samples used in evolutionary research (i.e. neutral buffered formalin and/or ethanol) alters the material properties, meaning it is often necessary to use previously defined material properties for other species (Peterson and Müller, 2018). Keeping the properties constant between specimens can allow a comparison relating solely to the morphological changes and the resulting stress patterns (Peterson and Müller, 2018). When using different material properties to perform FEA, the pattern of the stress and strain is similar to what it would be with accurately measured properties, but the quantitative numbers and magnitude will differ (Strait *et al.* 2005).

In addition, FEA modelling is often not properly validated by experimental testing (Korioth and Versluis, 1997). For example, Dumont, Piccirillo and Grosse (2005) used FEA for modelling bone stress during biting in bat skulls, a task that would have been difficult *in vivo* as it requires surgical placement of strain gauges within the mouth of the bats which could produce a negative impact on normal feeding behaviour. However, the authors stated that although FEA is an effective alternative, the results should still be compared with *in vivo* analyses of bone strain to confirm the results from modelling (Dumont, Piccirillo and Grosse, 2005). Similarly, to investigate how the skull of two species of Lake Malawi cichlids coped with stress during biting, Cooper *et al.* (2011) used FEA and reported that a species which had short faces with steeply descending profiles had a more robust neurocranium which was capable of handling this stress. Part of this study involved modelling the expected bite force of the jaws but because there was no data available from direct testing, an estimation of the bite force was used. While FEA can serve as a substitute for direct testing, it is likely to be more informative when combined with traditional simpler methods, and whilst some complications exist, proves a step in the right direction to understanding how form and function interact.

1.3 Reasons, Prevalence and Mechanisms for Sexual Dimorphisms

Differences between the phenotypes of males and females of a species are common in nature and defined as sexual dimorphism. ESD is a special case of sexual dimorphism and is thought to evolve when males and females face different selection pressures in shared, ecologically relevant traits. However, because the same genome must remain compatible in sexually reproducing species, this is often termed sexually antagonistic selection (Cox and Calsbeek, 2009). Sexually antagonistic selection has been explored in Soay sheep where horn phenotypes are subject to different selection pressures in males and females. Males with reduced horns (referred to as scurred) produce fewer offspring per year than those with normal horns, however in females, the scurred horn phenotype is advantageous (Robinson *et al*. 2006). Sexual dimorphism can however potentially overcome the genetic conflict caused by sexually antagonistic selection (Cox and Calsbeek, 2009).

Fortunately, investigations of ESD can be readily expanded through a set of criteria proposed by Selander (1972) to detect ecological causes for sexual dimorphism. These criteria suggest that sex-based modifications of the size or shape of trophic morphology, which would not be expected to result from sexual selection, is the most reliable way to conclude an ecological role in sexual dimorphism. However, Shine (1989) suggests that these criteria are too difficult to use in practice because it may exclude cases where there is ESD in traits other than the trophic morphology (for example, body size) or cases where the trophic morphology has diverged in response to ecology but also relates to reproduction. Any instances of sexual dimorphism related to reproduction that have not evolved under sexual selection, such as one sex using the mouth for nest building, could be considered ESD by Selander's (1972) criteria (Shine, 1989). For example, the buccal cavity volume of male coral reef cardinalfish (multiple species of the family Apogonidae) were found to be larger in males than females in five out of seven species investigated by Barnett and Bellwood (2005) and this was only attributed to the fact that males are mouth-brooders; this could be considered as ESD using the criteria above despite the fact that no

trophic aspect was considered. The function of the trophic apparatus, whether it be a foraging or reproductive role (or both), is not fully understood in cases of adaptive divergence. Recent work by tkint *et al*. (2012) investigated the trade-off between mouthbrooding and feeding performance in a 'biting' and a 'sucking' species of haplochromine cichlids. The authors observed a trade-off between feeding performance and mouthbrooding in the two species and suggested that there were potentially numerous selection factors acting on males and females during the African cichlid radiation events. It would also be challenging to conclude that differences in trophic morphology are solely due to ecological reasons if there are foraging and reproductive pressures that rely on the same anatomy. It is therefore possible that the criteria above are not applicable in practice (Shine, 1989), and that the evolution of ESD encompasses a mixture of factors relating to both ecology and reproduction with functional trade-offs between these factors playing a key role in the evolution of trophic traits.

Perhaps the most convincing examples of ESD in nature involve a series of studies in hummingbirds. Temeles and Roberts (1993) found that in rufous hummingbirds, female bills were 10.5% longer than males and that this is related to a difference in foraging ability; however, they concluded that despite strong evidence of ESD, reproductive factors and sexual selection may have also played a part in this dimorphism. Similarly, in hermit hummingbirds, the bill is 60% more curved in females than in males with evidence indicating that this is due to differential use of plants for food (Temeles, Miller and Rifkin, 2010). Finally, males and females of the purple throated carib hummingbird (*Eulampis jugularis*) feed from dimorphic *Heliconia* flowers that correspond to the shape and size of their bill (Temeles *et al*. 2000). In addition to the hummingbird examples, other taxa demonstrate evidence of ESD suggesting that it is potentially widespread in nature. For example, in *Anolis* lizards, there are sex differences in diet, behaviour and microhabitat use linked to differences in body shape (Butler and Losos, 2002; Butler, Sawyer and Losos, 2007). This is notable since *Anolis* are an exemplar system for the study of adaptive radiations. For other reptiles, sexual dimorphism in head size and shape is prevalent in snakes and is likely due to ESD as sex-based dietary differences have been recorded for

Arafura file snakes and twelve other species (Shine, 1991; Houston and Shine, 1993). These findings from hummingbirds, snakes and lizards strongly suggest a wider prevalence of ESD and support the need for research of ESD in the context of adaptive divergence.

Tests for the presence of ESD in fish have been especially rare. In skates (Rajidae), some species can display sexual dimorphism in tooth shape whereby female teeth are adapted for crushing whereas male teeth are adapted for tearing; these trophic differences are suggested to reduce foraging competition (Feduccia and Slaughter, 1974). Additionally, sticklebacks, which provide model examples of adaptive divergence, show evidence of ESD. Cooper, Gilman and Boughman (2011) reported that differences in head shape between sexes was greater than between eco-morphs and concluded that sexual dimorphism is likely linked to differences in feeding, but sexual selection may also be involved. Similarly, following an examination of ten populations of threespine sticklebacks, Aguirre and Akinpelu (2010) reported sexual dimorphism in trophic morphology suggesting niche divergence between the sexes. Differentiating between ESD and dimorphism due to sexual selection is challenging, however, investigating the functional aspects of dimorphic characteristics could help to distinguish between the two factors. Whilst clear and unambiguous cases of ESD are limited, the evolution of sexual dimorphism may actually be the result of multiple factors such as ecological divergence and sexual selection acting together (Bolnick and Doebeli, 2003).

1.4 The Genetic Basis of Adaptive Variation

Evolution is often defined as genetic change over time. Therefore, exploring the genetic basis of divergence in relation to morphology, and how this impacts on function and resource use, is key to understanding the evolutionary process of an adaptive radiation (Irschick *et al*. 2013). While it is important to elucidate the genes responsible, it is also of interest to know how the different morphologies initially arise. In terms of developmental timing, traits which are functionally simplistic are thought to develop early on compared to complex and integrated

morphological traits (Irschick *et al*. 2013). Therefore, examining the development of different morphological traits can improve our understanding of the evolution of function. Evolutionary change in function can be relatively simple; in the structures that make up the bat wing, small changes in expression of a handful of important genes during development and the evolutionary process of bats have resulted in large changes in bone morphology (Sears, 2008). Thus, there is a growing movement toward combined studies of development, function, and genetics within the context of adaptive divergence (Irschick *et al*. 2013).

So far, studies focused on determining the genetic basis of adaptation have largely implied functional changes without direct tests of "form to function". For example, both *Cam1* and *bmp4* genes have been identified to have a role in determining the shape of the mandible. Further study shows that both of these genes are involved in the early development of beak shape in finches, and in the mandible shape of cichlids (Abzhanov *et al*. 2004; Albertson *et al*. 2005; Parsons and Albertson, 2009). Furthermore, the *Cam* dependent pathway is likely to have been involved in the evolution of beak length (Abzhanov *et al*. 2006). *Cam1* is specifically predicted to play a role in the shaping and remodelling of the jaw (Parsons and Albertson, 2009). For *bmp4*, Albertson *et al*. (2005) concluded that it has a role in controlling the biting or crushing morphology of the cichlid mandible. Similarly, in finches, *bmp4* expression shows a strong association with deep and broad beaks used for crushing seeds (Abzhanov *et al*. 2004). Additionally, the *ptch1* gene has recently been shown to associate with adaptive changes in the mandible of cichlids (Roberts *et al*. 2011); it remains to be determined if this gene is important for morphological divergence in other adaptive radiations. Whilst we understand to a certain extent the roles of these particular genes, the genetic and developmental basis of adaptation is a route that needs to be explored in conjunction with tests of biomechanical function and how this relates to different morphologies.

Whilst genetic control of the jaw has been explored between species, this area has seldom been covered for sex. As stated previously, one reason is that it is difficult to provide evidence that ecological factors are responsible for

differences between the sexes. There is also a degree of genomic conflict involved in the evolution of sexual dimorphism whereby the same genes code for the same traits in both males and females (Leinonen, Cano and Merilä, 2011). Populations of three-spine sticklebacks show sexual dimorphism in certain traits; however, despite the fact that the genetic basis of these sexually dimorphic traits (body shape and armour) is similar in both sexes, this has not prevented their evolution. It has been reported that traits for body shape and size have been genetically mapped to the sex chromosome suggesting that differential gene expression between sexes for these traits could provide a resolution to genomic conflict (Leinonen, Cano and Merilä, 2011). Similarly, in Lake Malawi cichlids, there is a sexually antagonistic trait in the form of colouration; the orange blotch (OB) phenotype is advantageous to female fitness but not to males. To overcome this genomic conflict, rather than sexually dimorphic gene expression, Roberts, Ser and Kocher (2009) have suggested that this OB locus is under tight genetic linkage with an important female sex determining region. The problem of genomic conflict is important to the evolution of sexual dimorphism, how organisms overcome this will prove an important area to investigate with regards to understanding the genetic basis of adaptive variation and more specifically, ESD.

1.5 Environmental Influences on Adaptive Variation

Whilst elucidating the genetic basis of phenotypic traits is important, how the environment influences adaptive phenotypic variation is becoming a key area of evolutionary research. In the majority of studies exploring the relationship between the genotype and phenotype in natural populations, the percentage of variation in the phenotype that can be explained solely by the genotype is relatively small (Hu and Albertson, 2017). In addition to understanding the link between the genotype and phenotype, the field of evo-devo seeks to also understand how developmental processes can bias or constrain evolutionary change (Raff, 2000; Brakefield, 2006). Epigenetics, first described by Waddington (1942; 1957), includes factors above the level of the genotype that

can also influence how the phenotype develops (Jamniczky *et al*. 2010; Hu and Albertson, 2017). For example, the biomechanical environment an organism experiences during development can influence the resulting phenotype. This is the case for bone shape which is influenced by mechanical forces experienced over ontogeny (Young and Badyaev, 2007; Klingenberg, 2010). Similarly, recent work by Hu and Albertson (2017) demonstrated an epigenetic mechanism in adaptive craniofacial variation in Lake Malawi cichlids whereby different biomechanical conditions in the form of gaping behaviour of the larvae (both natural and experimentally induced) had an effect on craniofacial shape. Nonetheless, genes underlying traits are a major part of understanding the process of evolution, however it is clear that environmental effects during development are also crucial to phenotypic variability and adaptive divergence.

The production of multiple phenotypes from one genotype in response to variable environmental conditions, termed phenotypic plasticity, provides organisms the chance to respond to environmental variability and is therefore an important factor to consider with regards to adaptive divergence (West-Eberhard, 1989; Murren *et al*. 2015). Within the field of evo-devo, numerous theories for the role of phenotypic plasticity in adaptive radiation have been suggested. Dating back over a century ago, the Baldwin effect (originated by Baldwin 1896, 1902), represents a key theory of phenotypic plasticity whereby plasticity allows for adaptation to the environment and this variation is then acted on by the process of natural selection (Crispo, 2007). Additionally, genetic assimilation asserts that a plastic trait can become canalised when the environment stabilises with the trait expressed regardless of environmental conditions (Waddington 1953; West-Eberhard 2003). If the environment continues to vary, then this trait can remain phenotypically plastic, thus resulting in polyphenism within a population (Waddington 1953; West-Eberhard 2003; Parsons and Albertson 2009). The spadefoot toads (*Spea* sp.) display polyphenism and represent one of the most notable examples of adaptive divergence through phenotypic plasticity whereby either a carnivorous or omnivorous phenotype develops depending on prey density (Pfennig 1990; Pfennig and McGee, 2010). Given that fish and amphibian taxa which show polyphenism in response to competition for resources have a greater species

richness than those that do not, it has been suggested that phenotypic plasticity could be the key process that accelerates an adaptive radiation (West-Eberhard 2003; Pfennig and McGee, 2010; Pfennig *et al*. 2010)).

The role of phenotypic plasticity in the process of adaptive radiation has been a key topic of discussion in the especially within the context of the extended evolutionary synthesis (EES). The EES is an updated conceptual framework which posits that factors such as developmental bias and plasticity are key to how evolution progresses (Laland *et al*. 2015). The EES attempts to move away from the traditional gene-centric view and has been a recent, albeit controversial, topic of discussion within the field of evolution (see Laland *et al*. 2014). Indeed, the plasticity-first hypothesis is the idea that adaptive traits are generated and advanced by phenotypic plasticity; although this idea is somewhat controversial (see Levis and Pfennig (2016) for discussion). Adaptive radiation is the evolution of multiple species over a rapid time frame from an ancestral population in response to differing ecological conditions (Schluter, 2000). If environmental conditions change, plasticity can provide a rapid change in phenotype and therefore promote diversification (West-Eberhard 2003; Pfennig *et al*. 2010). Phenotypic plasticity could aid survival and provide a quick phenotypic response in new environmental conditions, therefore enabling the process of adaptive radiation to occur (Pfennig *et al*. 2010). The "flexible-stem" model proposes that plasticity in an ancestral group represents the phenotypes present in the subsequent adaptive radiation (West-Eberhard 2003; Pfennig *et al*. 2010). The flexible stem model has been supported in exemplar adaptive radiations including threespine stickleback (Wund *et al*. 2008), and the focus of this thesis, African cichlids (Parsons *et al*. 2016). In their paper, Wund *et al*. (2008) examined the plastic response of marine stickleback (the ancestral population) and reported a plastic response in phenotype similar to the freshwater benthic and limnetic ecotypes. It is evident that phenotypic plasticity could therefore play a key role in the radiation process.

Given that ESD is a type of adaptive divergence, it is logical to hypothesise that there could be dimorphism in phenotypic plasticity between sexes. In beetles, some traits relating to sexual selection, such as weapons and ornaments, exhibit

sexual dimorphism in plasticity which is controlled by links to sex-determining loci and the condition of the organism (Zinna *et al*. 2018). However, relatively few studies on phenotypic plasticity within the context of adaptive radiation consider this possibility. Sexual dimorphism in plasticity could have functional and ecological consequences if the trait in question is under divergent selection. In the case of ESD, in a trait that has ecological consequences it would therefore be logical to suggest that a difference in plastic response to a changing environment between sexes could contribute to ESD.

1.6 African Cichlids as an Evo-Devo Model

To examine adaptive divergence in relation to function, the cichlid fish which inhabit the East African Great Lakes are an excellent model as they show extensive craniofacial variation across their adaptive radiation (Cooper *et al*. 2010). This diversification has allowed for the exploitation of different environmental niches and food sources. These niche specialisations can include, but are not limited to, planktivores, insectivores and mollusc crushers (Albertson *et al*. 1999). Species are separated into two broad foraging categories; suction feeders who feed on mobile prey, and biters, who feed on hard prey (e.g. molluscs), or algae attached to rocks (Albertson and Kocher, 2006). Suction feeders are characterised by long, slender jaws whereas short, broad jaws are possessed by biters (Albertson *et al*. 2005). The two focal species of this thesis, *Labeotropheus fuelleborni* (LF) and *Tropheops* "Red Cheek" (TRC), are both algal feeders but differ in their feeding mode; LF are algal scrapers whereas TRC pluck and twist to remove strands of algae from rocks (Parsons *et al*. 2015; Albertson and Pauers, 2018). Both species are biting feeders, but the subtle differences in morphology and foraging behaviour mean they are an excellent choice for exploring finer scale patters of divergence (**Figure 1-1**; Parsons *et al*. 2015). As the extent and axis of morphological variation in this radiation is well known, they provide an excellent model to test ideas about form and function and how this relates to ecology.

The variation in craniofacial shape of African cichlids can be utilised for genetic studies because they are closely related and share a common genetic background (Powder and Albertson, 2016). This means they are an excellent laboratory model to test ideas about the genetic control of adaptive divergence (Albertson and Kocher, 2006; Streelman, Peichel and Parichy, 2007). Indeed, recent data has showed that Lake Malawi cichlids have a lower genetic diversity than laboratory reared zebrafish (*Danio rerio*) (Loh *et al*. 2008). This high phenotypic variation in conjunction with low genotypic variation means that African cichlids are an excellent choice for population genomics and quantitative trait loci studies to investigate the relationship between the genes linked to phenotypic variation (Powder and Albertson, 2016). Furthermore, as this variation is similar to craniofacial disorders in humans, a common birth defect, potentially understanding the genes involved in mandible shape and development in cichlids will not only enhance our understanding of evolutionary ideas, but could have a clinical benefit as well (Parsons and Albertson, 2009).

Sexual dimorphism is prominent in African cichlids, but there has been comparatively little investigation into ESD. So far, most research has focused on sexual dimorphism in body size and of the few studies which consider sexual dimorphism of trophic morphology, the focus has been on how this relates to reproduction and sexual selection (e.g. Oliveira and Almada, 1995; Herler *et al*. 2010). Recently, Parsons *et al*. (2015) reported sexual dimorphism in craniofacial shape in the F2 generation of LF and TRC hybrids and suggested that this dimorphism has been an important part of the Lake Malawi cichlid radiation as it aligns with the divergence between species, and the adaptive radiation as a whole. This evidence suggests ESD needs further investigation in African cichlids. Notably, Lake Malawi cichlids use their mouths for specialised foraging, while females also use their oral cavity to brood their young. This provides a key element for cichlids to contribute to our understanding of ESD, as sexes are likely to differ in their degree of trade-off between foraging and reproductive uses for their mandible; this is also pertinent to the idea of sexual dimorphism in plastic response. Comparisons between males and females in shape, function, and plasticity could therefore be indicative of ESD and perhaps enhance our understanding of how adaptive radiations occur.

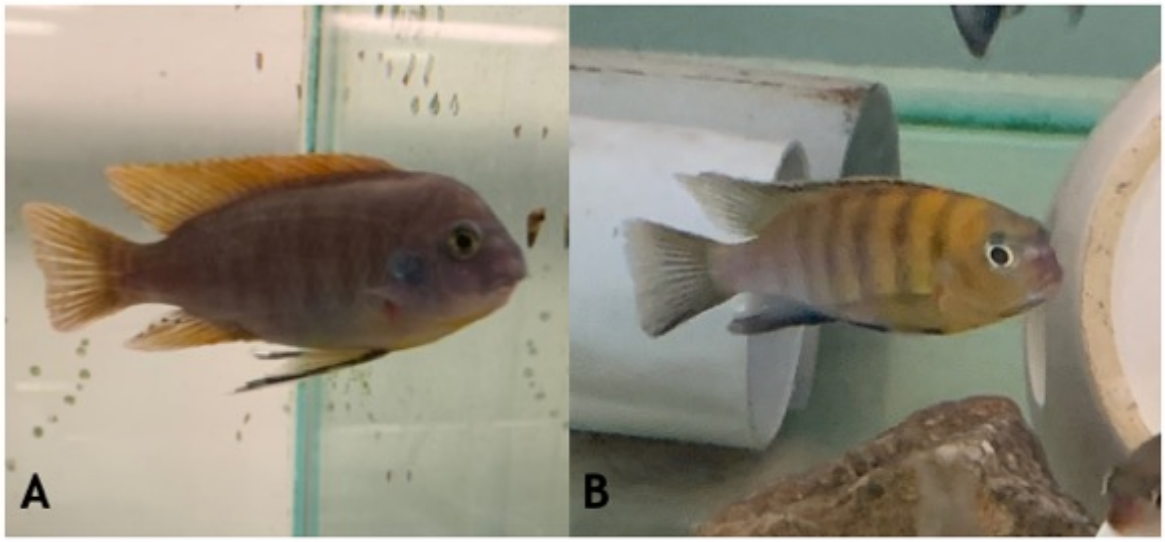

Figure 1—1: The two focal species of this thesis: a) *Labeotropheus fuelleborni* and b) *Tropheops* "Red Cheek". Photographs taken in the University of Glasgow aquarium facilities.

1.7 Conclusions

To conclude, the genetic basis of shape and function in the craniofacial region of examples of adaptive radiation have yet to be determined in detail. Adopting an approach which combines the biological questions with functional techniques should provide more insight into these processes. Traditionally in the study of adaptive divergence, functional morphology studies have been overlooked in favour of a more simplistic methods to relate form to ecology. It is timely that the study of adaptive divergence and ESD move toward integrative studies of how form, function, genes and ecology are interrelated as this would provide a greater insight into the evolutionary process. African cichlids are an excellent evolutionary model which can be tested in the laboratory and as such, they provide a way to test evolutionary ideas and questions about adaptive divergence and ecological sexual dimorphism that may be applicable to other forms of adaptive radiation.

1.8 Outline of Thesis

In **Chapter 2,** I use an F2 hybrid population (from LF and TRC) to investigate the genetic basis of mandible shape using μ-CT scanning in combination with 3D geometric morphometrics and genotype information. I test for sexual

dimorphism in mandible shape and use quantitative trait loci (QTL) mapping to investigate candidate genes of interest involved in the adaptive divergence of the mandible. I then use whole mount *in-situ* hybridisation (WISH) to test for evidence of differential candidate gene expression between LF and TRC at a crucial point of development of the mandible.

In **Chapter 3,** I use 3D morphometrics to investigate the morphology of TRC and LF to test for sexual dimorphism in mandible shape in the two parental species of this thesis to complement the data presented in **Chapter 2**. In addition, I use finite element analysis (FEA) to explore how the mandible copes with loading which could be encountered during foraging to test questions about interspecific and intraspecific divergence using finite element meshes created from 3D models of TRC, LF and F2 mandibles.

In **Chapter 4,** I investigate phenotypic plasticity in craniofacial shape with a diet treatment experiment, morphometrics and then assessments of functionally relevant traits to approach phenotypic plasticity from a novel angle by considering whether there is a sexually dimorphic aspect to phenotypic plasticity in African cichlids.

Chapter 2: Exploring the Genetic Basis of Adaptive Divergence in the Cichlid Mandible

2.1 Abstract

To enhance our understanding of the process of adaptive radiation, it is necessary to consider a range of contributions from different levels toward divergence patterns. In this chapter, the genetic basis of phenotypic divergence is explored using an F2 hybrid cross between *Labeotropheus fuelleborni* and *Tropheops* "Red Cheek". These two Lake Malawi cichlids occupy a similar ecological niche but use different tactics to exploit food. While most studies in this context would only focus on divergence between species, this chapter also considers the possibility of ecologically relevant sexual dimorphism. Using a novel method in combination with geometric morphometrics, patterns of shape variation in the mandible were quantified and tested for associations with genotypic variation through a quantitative trait loci (QTL) mapping approach. The major axis of shape variation related to the width of the jaw in the F2 hybrids and this was likely attributable to species differences, however there was also strong evidence of sexual dimorphism in mandible shape which could be ecologically relevant. Following on from the subsequent QTL mapping, population genomic data confirmed that a QTL region containing the candidate gene *zeb1* was subject to selection in this cross. With regards to sexual dimorphism, a QTL on the sex determining loci for these species (LG7) appeared in the majority of these models suggesting a key role for sexual dimorphism in the evolution of the cichlid mandible. The results presented in this chapter suggest that the Lake Malawi radiation is likely to be comprised of multiple levels of variation which could explain the success of the adaptive radiation.

2.2 Introduction

A central focus for evo-devo is to gain an understanding of the mechanisms that generate adaptive variation (Hendrikse, Parsons, and Hallgrimsson, 2007). Adaptive radiations, whereby multiple species evolve from a common ancestor adapted to different ecological niches, are ideal for exploring such mechanisms (Schluter, 2000; Irschick *et al*. 2013). However, the emergence of new species through ecological processes is unlikely to be an instantaneous process. Therefore, both interspecific and intraspecific phenotypic variation can inform our understanding of adaptive mechanisms. Indeed, for Darwin's finches, the major axis of divergence between species relates to beak morphology which ranges from long, shallow and pointed beaks to short, deep and blunt beaks, but there is also subtle, significant variation in beak shape nested within species on the same island (Foster, Podos and Hendry, 2007). Therefore, such variation could initiate adaptive radiations and represent an important level of biodiversity (Parsons *et al*. 2015).

Adaptive radiations commonly involve change in trophic morphology in relation to ecological niche. Such changes determine the type of food consumed, and the efficiency with which it is processed (Parsons and Albertson, 2009). Craniofacial variation has been especially notable for this within the adaptive radiations of African Rift valley cichlids. Specifically, cichlids diverged along a morphological axis common to a variety of fish taxa with short jaws evolving for biting specialists who feed on hard prey, and the evolution of longer jaws for suction feeding on mobile prey (Albertson *et al*. 2005). Within this main axis of Malawi cichlid divergence lies smaller differences in feeding techniques between species with a similar ecology (Ribbink *et al*. 1983; Albertson, 2008; Parsons *et al*. 2015). For example, several species focus on foraging of algae, but using differing tactics. A likely key to this type of divergence are functional changes in the mandible regarding its shape and structure (Westneat, 1995).

The mandible itself represents a major vertebrate innovation and is usually among the first areas of contact with prey. This makes movement of the mandible especially important for ecological success and it is likewise a site of

major muscle attachment within the head (Kassam, Adams and Yamaoka, 2004; Conith, Lam and Albertson, 2019). Understanding variation in such craniofacial traits have in turn been a focus of a number evo-devo studies with several genes being implicated for the production of adaptive variation (e.g. *bmp4, caM1, ptch1* and *Wnt)* (Albertson, Streelman and Kocher, 2003a; Abzhanov *et al*. 2004, Abzhanov *et al*. 2006; Roberts *et al*. 2011; Hu and Albertson, 2014, 2017; Liu, Rooker and Helms, 2010; Parsons *et al*. 2014). Surprisingly, the mandible itself has rarely been the focus of such studies on the genetic architecture of adaptive phenotypic variation. Given its vital role in feeding, gaining an understanding of what determines variation in this structure can greatly contribute to our understanding of adaptive processes.

An emerging view within adaptive divergence research is that sexual dimorphism can represent an important level of variation. Specifically, it is now apparent that differences between sexes can evolve in relation to alternate ecological conditions resulting in "ecological sexual dimorphism" (hereafter ESD) (Shine, 1989). This raises the notion that ESD could be a key feature of many adaptive radiations. However, males and females generally share the same genetic variation within a population resulting in genomic conflict which perhaps limits their specific evolutionary responses (Cox and Calsbeek, 2009). Nonetheless, sexual differences in reproductive effort could result in adaptive divergence of ecological traits (tkint *et al*. 2012). While some clear examples of ESD exist in nature, in most cases disentangling the cause of sexual dimorphism is challenging. For example, sexual dimorphism is most often thought to evolve as a result of sexual selection, such as through secondary sexual characters or through differing demands on reproduction and life history, rather than through demands on foraging ecology. This means that the contribution of ESD to adaptive radiation is relatively unknown. Taking the traditional view, theoretical models show that sexual dimorphism can evolve exclusively from ecological selection pressures (Slatkin, 1984); it should be more widely considered that ecological divergence could interact with sex (Bolnick and Doebeli, 2003).

Currently, ESD is not widely studied within the context of adaptive radiations likely because both types of divergence are considered "two sides of the same

ecological coin" (Bolnick and Doebeli, 2003; Cooper, Gilman and Boughman, 2011). In other words, this means that ESD and traditional ecological divergence cannot co-occur because they result from the same ecological conditions (Bolnick and Doebeli, 2003). Contrasting this idea are a number of empirical examples of ESD that occur in the presence of broader patterns of adaptive divergence. For example, Cooper, Gilman and Boughman (2011) reported sexual dimorphism in adaptive craniofacial shape variation for threespine sticklebacks (*Gasterosteus aculeatus*) that exceeded differences between ecological species. Additionally, McGee and Wainwright (2013) reported sexual dimorphism in the kinematics of the trophic apparatus in anadromous threespine stickleback, in particular jaw protrusion, which has been shown to affect suction-feeding performance (e.g. Schaeffer and Rosen 1961; Motta 1984; Holzman *et al*. 2008; Holzman *et al*. 2012). Such sexual dimorphism is likely functionally and ecologically relevant as differences in jaw protrusion are often identified in cases of divergence in fish (Motta 1984; McGee and Wainwright, 2013). Indeed, McGee and Wainwright (2013) suggest that sexual dimorphism in feeding mechanics could facilitate rapid divergence in a novel environment by anadromous stickleback populations because small-scale ecologically relevant variation already exists between the sexes. The traditional view of ESD is that it is more likely if the trait in question is exempt from sexual selection, however in sticklebacks it is clear that both ecological and reproductive differences persist between the sexes and these aspects could have acted together in the evolution of sexual dimorphism (Shine, 1989, 1991; Bolnick and Doebeli, 2003). Therefore, for the Lake Malawi cichlid radiation, intraspecific variation in trophic morphology could be both ecologically and reproductively important, and in combination with interspecific differences, has potentially contributed to their explosive radiation.

This chapter investigates the genetic basis of shape variation in the mandible in high resolution while also assessing aspects of sexual dimorphism in relation to ESD. Ecologically relevant sexual dimorphism in craniofacial shape has been characterized in cichlids along with general sexual dimorphism in body size, colouration and reproductive effort (Kocher, 2004; Parsons *et al*. 2015; McWhinnie and Parsons, 2019). Given that Lake Malawi females are

mouthbrooders, I predicted that this could impose constraints on their ability to utilise the same food sources as males and lead to sex-based phenotypic differences in the mandible. I also expected that males would have a wider mandible than females as previous work indicates that males have a phenotype adapted for biting more than females, and a wider mandible confers a greater biting advantage (Parsons *et al*. 2015; McWhinnie and Parsons, 2019). If sexual dimorphism follows the trend of divergence within the radiation this would suggest ecological relevance in line with ESD.

For sexual dimorphism to evolve, resolutions to the genomic conflict it causes are needed. Therefore, QTL mapping which takes both species and sex differences into account could highlight new areas of interest in the genome especially in light of ESD. Specifically, if sexual dimorphism is important to adaptive divergence in the mandible I would then expect to find QTL on LG7 as this is the hypothesised sex-determining region in cichlids (Ser, Roberts and Kocher, 2010). Close physical linkage to sex determination loci on a chromosome likely provides an easier target for selection to overcome sexual conflict when there are nearby loci that can provide adaptive value as is the case for certain sexually dimorphic colour patterns (Roberts, Ser and Kocher, 2009). However, through epistatic interactions with sex in the genome, some distantly located loci may also form targets for selection that resolve sexual conflict. Therefore, I also expected to find different QTL when sex was modelled as a covariate which could indicate areas of the genome undergoing selection from sexual conflict.

2.3 Methods

2.3.1 Details of the F2 intercross

To examine the genetic basis of mandible shape, an F2 experimental cross between two species of Lake Malawi cichlids was used. Whilst they occupy a similar ecological niche, both parental species exhibit distinct differences in mandible shape; *Labeotropheus fuelleborni* (LF) has a wide, short jaw for scraping algae whereas *Tropheops* “Red Cheek” (TRC) has a short, narrow jaw

for plucking algae off of rocks (Parsons *et al*. 2015; Navon, Olearczyk and Albertson, 2017). Both are "biting" feeders but do this in different ways meaning they are an excellent for exploring finer scale patterns of ecological divergence. LF are highly specialised feeders that scrape algae whereas TRC "nip" and "pluck" algae from rocks (Parsons *et al*. 2015; Albertson and Pauers, 2018). Specifically, an LF female from Makanjila Point was crossed with a TRC male from Chizumulu Island with F1 siblings interbred to create F2 individuals. The F2 were initially reared in 10-gallon tanks and then moved into 40-gallon tanks to accommodate growth when they were between one and two months old. Fish were reared until sexual maturity and then euthanised, fixed in 10% buffered formalin, and stored in 70% ethanol. Sexing was based on external colouration, vent size and internal dissection in a subset of individuals. Further details on rearing can be found in Parsons *et al*. (2015).

2.3.2 µ-CT Scanning of the mandible

Shape variation was quantified in the mandibles of F2 hybrids through a series of steps. First, the mandible was isolated by disarticulation from the upper jaw with surrounding tissue being carefully removed to allow detachment from each specimen (n = 244). The jaws were then rehydrated by stepping them through different concentrations of ethanol solution (70%, 50% and 25%) for transport to the University of Glasgow where they were transferred and stored in 1X PBS. To allow for the quantification of 3D shape, each mandible was subjected to µ-CT scanning using a Bruker Skyscanner machine (model 1172; Bruker, Billerica MA) located at the University of Strathclyde, Glasgow, UK. Each mandible was scanned individually using a standard procedure that included removal from the storage solution approximately 5-10 minutes before scanning. This ensured that mandibles were hydrated and "wet" for the scanning process to obtain a model that was as realistic as possible to *in vivo* conditions. Mandibles which had separated down the midline during removal, in transit, or when handled during the scanning process were not scanned (n = 43). Across specimens, the parameters of the µ-CT scans were kept constant at 70kV and 10 µm resolution using the largest camera. This resolution provided an appropriate balance

between the quality of the scan, file size and time management. To ensure that each slice was aligned properly after scanning, raw images were reconstructed using NRecon (version 1.6.9.18); each model was reconstructed separately but the reconstruction settings (smoothing, ring-artefacts reduction, and misalignment correction) remained constant across specimens.

2.3.3 3D Model Generation

3D models of mandibles were created for shape analysis using the software ScanIP (Version 7.0; available at: https://www.simpleware.com/). Specifically, for each specimen a stack of .bmp image files was loaded into ScanIP and a "Pixel Skip" value of 3 was chosen for each axis to reduce the file size of the raw data to a manageable value (from an initial 500-750MB to between 15-20MB per specimen). While such down sampling reduces the number of triangles in the model, this also reduces detail. Once the .bmp stack was loaded, the "Recursive Gaussian" filter was used at a value of 1.0 on each axis to remove noise from the model and to smooth the appearance. Following smoothing, the "Interactive Threshold" function was used to highlight the region of interest relating to bone density values recorded by the scanning process. Upper and lower threshold boundaries based on greyscale values were set manually to define the area of interest for each scan. The "Flood Fill" function was used to create a mask that included only the connected areas on the model to remove excess scanning artefacts. To create a model, this mask was then classified as a new surface model and a series of options had to be considered. The "Smart Mask Smoothing" option was chosen to apply topology and volume preserving smoothing functions. Then "Decimate and Reduction in %" was selected to reduce the number of triangles in the model to (~300,000) to keep the triangles consistent across models and further reduce the file size of the output. The model was then exported and saved as an STL (stereolithography) file and loaded into the software MeshLab (available at: http://meshlab.sourceforge.net/) to export as a .ply file. All models were created to the same coordinate scale to ensure consistency.

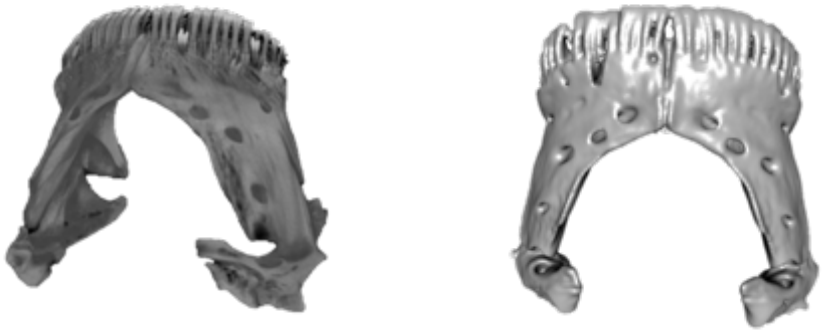

Figure 2—1: Processing of a 3D model of an F2 hybrid specimen. In the left panel, an unprocessed image 'stack' from the μCT data stack is shown. The right depicts a completed model as viewed in ScanIP.

2.3.4 Morphometrics of Mandible Shape

Mandible shape was quantified in 3D using a two-stage process. First, each model (represented as a .ply file) had a set of 20 landmarks (as in **Figure 3-2**) manually placed on homologous points of the surface using Landmark Editor (Available at: http://graphics.idav.ucdavis.edu). Landmarks were chosen to reflect the shape of the whole mandible based on previous studies (e.g. Albertson and Kocher 2001 and Parsons, Marquez and Albertson, 2012) and were saved as a pts (points) file. All data was then imported and processed with packages run within R version 3.4.1 (R Core Team 2017). Second, to increase detail of localised shape variation, a novel method was employed using Face3D (unpublished R package: Bowman, Vittert and Katina, *in prep*). This method, based on the principle of the "shape index" (Koenderink and van Doorn, 1992), automates the process of landmark placement using the topography of the surface itself to create a curve comprised of numerous points using anatomical landmarks as boundary points.

Using the initial set of 20 landmarks (used in **Chapter 3**), curves were created using the landmarks as start and end boundary points. After the landmarks were selected, the shape index algorithm was then run on the 3D model to create curves between two landmark boundary points by following the topography of the surface. Due to the complex nature of the surface of the mandible, the curves were tested to ensure they could be reliably created across all samples. As this was not the case, the five best curves were selected. This resulted in a final data set of 5 curves (using 10 landmarks from the initial set as the bounding

points) and 10 remaining landmarks (3, 6, 7, 8, 9, 12, 15, 16, 17 and 18 from **Figure 3-2**) across the entire model surface for each mandible (**Figure 2-2**). Each curve ranged between 20 to 100 points and this was homologous across samples.

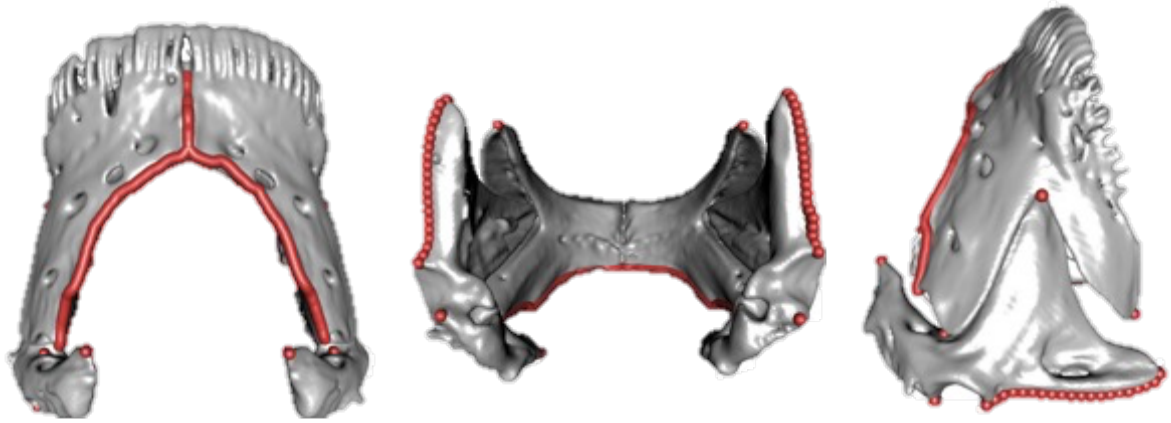

Figure 2—2: Landmarks and curves used for the morphometrics analysis. Landmark points were placed on the surface in landmark editor and the curves were created using Face3D. Mandible anatomy is described in more detail in **Chapter 3**.

In some samples damage made bilateral placement of some landmarks and curves challenging. Therefore, to complete landmark placement approaches were used on some samples (around 30%) to restore bilateral symmetry by mirror image reflecting parts of the 3D model (Gunz *et al*. 2009; Mitteroecker and Gunz, 2009, Zelditch, Swiderski and Sheets 2012b). To achieve this any missing landmarks/curves were filled in based on their bilateral homologues using custom code based on functions from StereoMorph (Olsen and Westneat 2015; Olsen and Haber 2017). Models with many parts missing, or parts missing on both sides, could not be accurately reflected or the curve algorithm did not work accurately, and so were removed from the dataset (n = 25). Once missing landmarks were added to create a "complete" model, the symmetric variation of the shape was extracted for each specimen using code from Face3D. Following these corrections, a Procrustes superimposition was performed using the *gpagen* function in geomorph on the landmark data to rotate, transform and scale to a common centroid size and orientation (Zelditch, Swiderski and Sheets 2012a; Adams and Otárola-Castillo, 2013). I then minimized allometric effects on

shape using a multivariate regression of Procrustes coordinates on geometric centroid size using the *procD.allometry* function in geomorph (Adams and Otárola-Castillo, 2013; Adams, Collyer and Kaliontzopoulou, 2019).

2.3.5 Statistical Analysis of Mandible Shape

To determine major trends in mandible shape for F2 hybrids, I employed a principle components analysis (PCA). To represent the relative proportion of variation explained by PCs I produced a scree plot which indicated a drop in explained variation after the first four PCs (see Zelditch, Swiderski and Sheets 2012a). Therefore, to test the effects of sex on F2 hybrid shape, an ANOVA was then conducted for each of the first four principal components (accounting for 80% of the shape variation). Sexual dimorphism in mandible shape was also assessed using a discriminant function analysis (DFA). Although selection of PC scores to include can be an arbitrary process, a suggested "rule of thumb" for discriminant analysis is to divide the sample size by 4 to get an indication of how many to use (Zelditch and Swiderski, 2018). Therefore, this analysis was conducted on principal component scores representing 99% (PC 1-44) of the shape variation with sex as a grouping variable using the MASS package (Venables and Ripley 2002).

2.3.6 Genotyping and Linkage Map Construction

To facilitate genetic mapping, genotyping was conducted on F2 hybrids; further details can be found in Parsons *et al.* (2015). Briefly, restriction site-associated DNA sequencing (RAD-Seq) was applied and reads were aligned to the reference *Maylandia zebra* version 0 genome (Brawand *et al.* 2014); further details of this work, and a resultant genetic map, can be found in Albertson *et al.* (2014). Additionally, RAD-Seq was applied to wild-caught LF and TRC to inform our genetic map of population genomic data. Briefly, initial sequencing identified 42,724 SNPs and a linkage map was created using SNPs with an Fst value greater

than 0.57 as this indicates a high level of divergence between populations in cichlids, making it likely that the quantitative trait loci (QTL) findings would be evolutionarily relevant (Mims *et al*. 2010, Parsons and Albertson 2013). This resulted in a genetic map of 946 loci over 24 linkage groups (numbered according to Lee *et al*. (2005)), and a map size of 1453.3 cM.

2.3.7 Quantitative Trait Loci (QTL) Analysis

To assess potential relationships between genotypes and phenotypic variation I conducted a quantitative trait loci (QTL) analysis using the qtl (Broman and Sen, 2009) and shapeQTL (Navarro 2015) package in R 3.4.1 (R Core Team 2017). This involved two main approaches including multiple QTL mapping (MQM) following the approaches of Broman and Sen (2009) and Arends et al. (2010), and multivariate QTL mapping following Maga et al. (2015) and Navarro and Maga (2016). Both methods are related in that they rely on a similar statistical approach. MQM mapping within the qtl package offers an automated procedure combining regression and interval mapping (Arends et al., 2010). The shapeQTL package offers Haley-Knott regression QTL mapping and is an extension of the qtl package that has been specifically created for multivariate shape data.

MQM was carried out on PCs 1 and 2 as they represented the highest proportion of variation in the dataset. To begin, standard interval mapping was conducted to identify QTL with LOD scores greater than 1. These QTL were then iteratively tested as cofactors in subsequent QTL models and kept or removed on the basis of their ability to improve the overall model. Permutation tests (n = 1000) were then run for each model to provide a genome-wide threshold LOD score at the 90% and 95% significance level. For significant QTL, locations were refined to the 95% confidence interval using the *bayesint* function in r/qtl. This function calculates the approximate Bayesian credible interval from the output of the QTL mapping and provides a range on the chromosome where the QTL is likely located. This range can then be used for further investigations such as candidate gene searching.

As shape is a multivariate trait, I also performed a quantitative trait loci analysis using a multivariate approach that included models both with and without sex as a covariate. This approach allowed for the effects of sex on the genetic architecture of mandible shape to be discerned. The first multivariate tests included the first two principal components representing 44% and 21% of the variation respectively. The next tests were conducted using the first four PCs and represented variation before my previous scree plot levelled off (Zelditch, Swiderski and Sheets 2012a) and accounted for approximately 80% of the variation. After PC10 the proportion of variation explained by each additional PC decreased to below 1%. Therefore, to maximize shape variation without further complicating the model, the final test included the first ten PCs accounting for 90% of the total variation.

Effect sizes, or percentage of the variation explained by the QTL, were calculated for the models. For the multivariate models, the effects sizes were calculated using the functions *fitqtlShape* and *effectsizeShape* which compute the regression projection scores of the qtl vector and calculates the percentage of variation explained by the qtl (Navarro *pers. comm.*). Effect sizes from the MQM models were calculated using the following equation (Parsons *et al.* 2016):

$$\Sigma(1 - (10\hat{} - ((2/n) * (LOD))))$$

Equation 2-1: The formula used to calculate the effect sizes of the MQM qtl whereby *n* is the sample size (176) and *LOD* is the logarithm of the odds score from model.

2.3.8 Population Genomics, Fine Mapping and Candidate Gene Searching

To increase the precision for locating candidate genes, population genomic data using wild-caught LF (n = 20) and TRC (n = 20) at 42,724 SNPs was used in tandem with QTL results. To identify locations under the greatest degree of selection within the 95% confidence interval of the QTL, markers with the highest Fst values were identified as this indicates regions undergoing particularly strong selection and are therefore divergent between species. As

LG18 appeared on multiple models from both the MQM and multivariate approach, this was selected for follow-up work. Fine-mapping using population genomic data narrowed down a region within the 95% QTL confidence interval, which ranged between positions 0-21 cM (depending on the model) spanning 14 of the population genomic markers.

Candidate genes found within this region were then identified using cichlid genome browsers (http://cichlid.umd.edu and http://em-x1.gurdon.cam.ac.uk) with the search region around each marker limited to 5kbp. When a gene was located near a QTL, cross-referencing with the online databases Ensembl (https://www.ensembl.org/index.html) and UniProt (http://www.uniprot.org) was conducted to identify details of the genes and assess their relevance to mandible, craniofacial shape, or bone development. To assess shape changes at the nearest markers closest to the top candidate identified from the QTL analysis, a DFA was conducted on principal component scores representing 99% (PC 1-44) of the shape variation with genotype at the nearest marker to *zeb1* (from the PC1 and PC2 MQM models) as a grouping variable again using the MASS package in R (Venables and Ripley 2002). In this cross, the AA genotype represents LF and the BB represents TRC.

2.3.9 Follow up: Investigating Candidate Gene Expression

To explore the anatomical location of expression for the candidate gene *zeb1* and investigate potential interacting genes, whole-mount *in situ* hybridisation (WISH) was used. I chose a key point in development when the mandible is beginning to form and genes that likely interact with *zeb1* are expressed (stage 16, approximately 4-5 days post fertilisation (Fujimura and Okada, 2007). Three genes were selected for WISH: *zeb1*, the candidate gene for mandible shape identified in this chapter; *bmp4*, a craniofacial candidate gene already identified as being expressed in cichlids at this stage (Albertson *et al*. 2005); and *col1a1* which is an osteogenic marker expressed where bone is beginning to develop (Hu and Albertson, 2014). Both *bmp4* and *col1a1* were selected as they are active during jaw development in cichlids therefore if *zeb1* was expressed at the same

time in a similar location, this would increase the evidence for *zeb1* being a suitable candidate involved in mandible development.

Probes were designed using custom forward and reverse primer sequences for *zeb1* and *bmp4* in Primer3 (available at: http://bioinfo.ut.ee/primer3-0.4.0/); the probe sequences from Navon, Olearczyk and Albertson (2017) were used for *col1a1*. The primers (listed 5' to 3') used to create the probes were as follows:

bmp4 F: AATATGCCAAGTCCTGCTGG	*bmp4* R: CACCCGACTGTAGCCGATAA
Col1a1 F: GCGGTGAGTACTGGATTGGT	*Col1a1* R: CCTCGGCTCTGATCTCAATC
zeb1 F: *TCGGTAGGAACAGGTGGAAC*	*zeb1* R: GTCACAGGCTTGCACTCAAT

Probes were synthesised first through a polymerase chain reaction (PCR) with cichlid cDNA (converted from RNA which was extracted from embryos between 4 and 6 days old) before being transcribed and precipitated into RNA using a mix containing RNA polymerase and digoxigenin (DIG) which binds to the probe. The protocol followed for WISH was based on methods described by Albertson *et al.* (2005) and Jacobs, Albertson and Wiles (2011). Embryos were collected at stage 16 (approximately 25 TRC from three different broods and 20 for LF from two different broods, divided between each probe and the control), euthanised and stored in 4% PFA for up to 7 days, before dehydration through a methanol series and stored at -20°C.

For the WISH, embryos were then rehydrated through the methanol series and digested with a 40μg/ml solution of proteinase K. Following digestion, embryos were then re-fixed in PFA and left in pre-hybridisation solution at 70°C. Pre-hybridisation solution was then switched for the probes and embryos incubated at 70°C overnight. The control embryos were taken through the exact same protocol but did not receive the probe at this stage and were instead left to incubate overnight in pre-hybridisation solution. The next morning, the probes were removed and placed back into storage and the embryos were taken through a series of washes with varying concentrations of pre-hybridisation solution and saline sodium citrate solution (SSC) at a constant temperature of 70°C. Following this, embryos were left to rock at room temperature for up to 3 hours in a

blocking solution comprised of animal serum, blocking reagent and maleic acid buffer (MABT). Following this, embryos were then blocked in solution containing Anti-DIG antibody overnight at 4°C. After blocking, embryos were washed six times in TST solution at pH 9 and stored in TST overnight at 4°C. Then, embryos were washed twice in NTMT at pH 9 and then embryos were added to a staining mix (20μl of solution in 1ml of NTMT) and the plate was covered with tinfoil and left to rock until colour developed. The colour reaction was stopped by washing in PBS and embryos were dehydrated using a methanol series to reduce background staining. Embryos were then rehydrated and cleared using varying concentrations of glycerol and PBST for a day before photographing in 75% glycerol using a dissecting microscope (Leica M165, Leica, Wetzlar, Germany) mounted with a digital camera (Leica DFC450 C, Leica, Wetzlar, Germany).

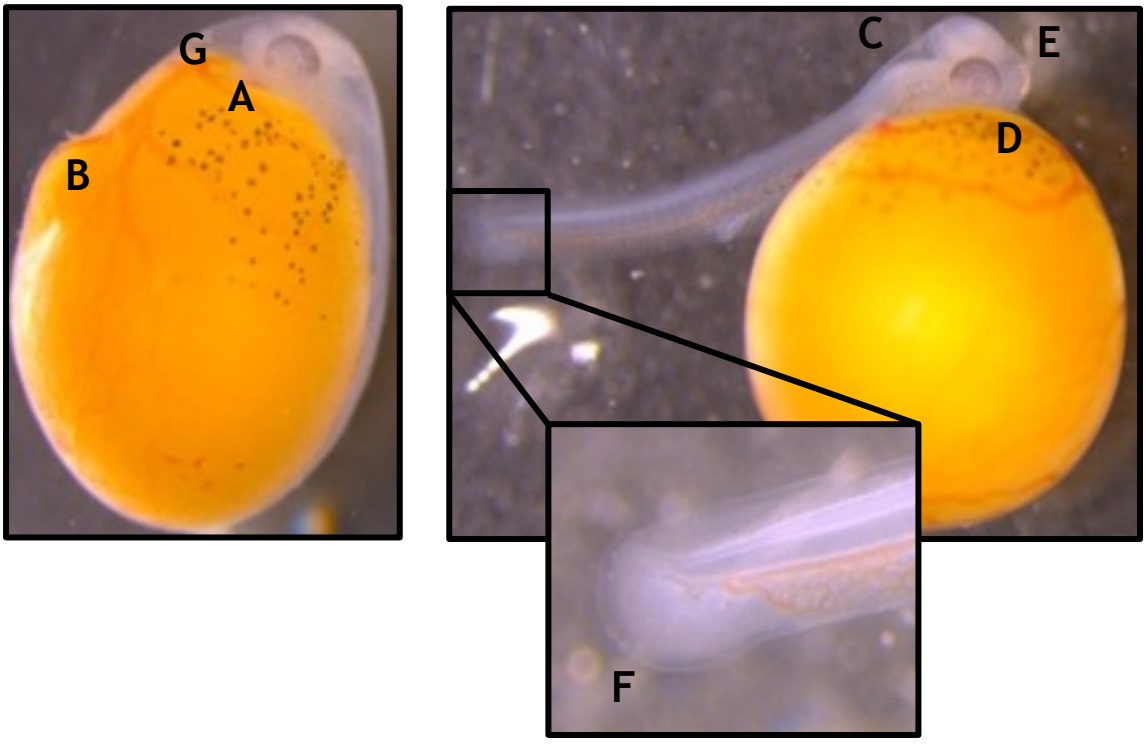

Figure 2—3: Embryos at stage 16 corresponding to 4-5 dpf; based on the staging of Nile Tilapia (*Oreochromis niloticus*) by Fujimura and Okada (2007) and an adapted staging guide for Lake Malawi cichlids (Albertson pers. Comm.). All larvae were the result of natural matings in the aquarium facilities at the University of Glasgow. Key features: heart beat (a); circulation (b); head is lifted (c); no jaw (d); darker eye pigment (e); no caudal fin rays (f). As hatching occurs between late day 3 and early day 5, some embryos may still be inside the chorion (g).

2.4 Results

2.4.1 3D Morphometrics

From the ANOVA, sex had a significant effect on PCs 2 and 4 but not PCs 1 or 3 (**Table 2-1**). The shape changes across PC1 resembled those of the two parental species; at the positive end of the axis the mandible was shorter and wider (like LF), whereas at the negative end the mandible was relatively longer and narrower (like a TRC) (**Figure 2-4**). Although there was considerable overlap in PC2 scores for each specimen, the negative scores were mostly male with the mandible being wider at the anterior but becoming relatively narrower at the posterior for females (mostly positive scores). For the discriminant function analysis correct classification for males was 87% and for females 83% (**Figure 2-5**). Along the LD1 axis there were subtle differences in mandible shape with males (negative scores) having broader and more "U" shaped mandible relative to females (positive scores) which had a more "V" shaped, narrower mandible (**Figure 2-5**). The female mandibles were also wider than the males between the articular web on either side (**Figure 2-5**).

Table 2-1: Results from ANOVAs conducted for the first four PC scores to test whether sex could influence mandible shape variation. The percentage of shape variation explained by each PC score is noted in brackets next to the model.

Model	DF	SS	F value	P value
PC1 (44%) ~ SEX	1	0	0	0.99
Residuals	174	0.25		
PC2 (21%) ~ SEX	1	0.008	13.29	**<0.001****
Residuals	174	0.11		
PC3 (8%) ~ SEX	1	0.00084	3.189	0.076
Residuals	174	0.045		
PC4 (7%) ~ SEX	1	0.00379	18.92	**<0.001****
Residuals	174			

**** P < 0.001**

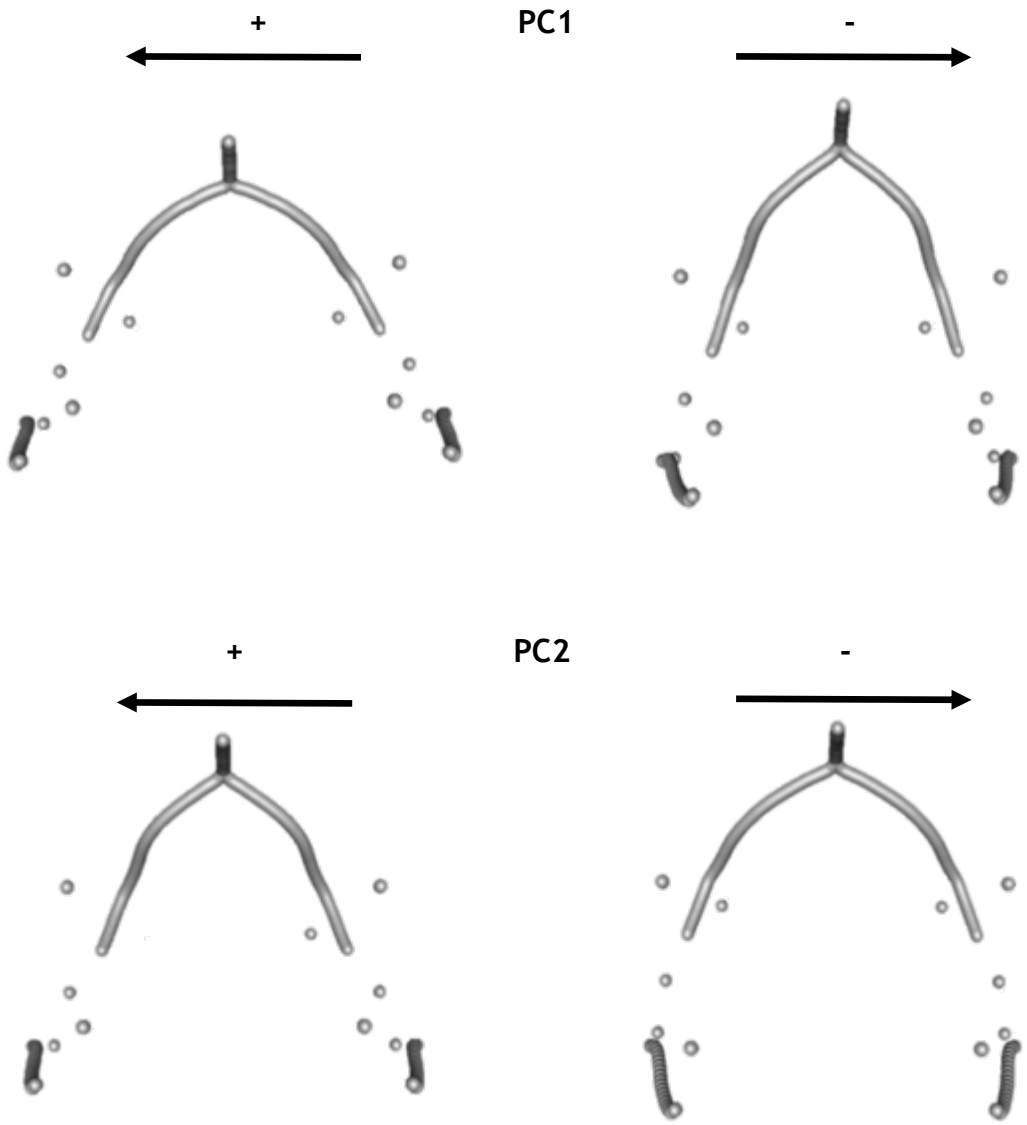

Figure 2—4: Variation in mandible shape explained by the first two PC axes from the dorsal view. PC1 is depicted in the upper panel and shows that negative scores result in a wide mandible relative to positive scores. In the lower panel PC2, which was affected by sex shows that a positive score corresponds to a narrower mandible (mostly females) relative to the negative scores (mostly males).

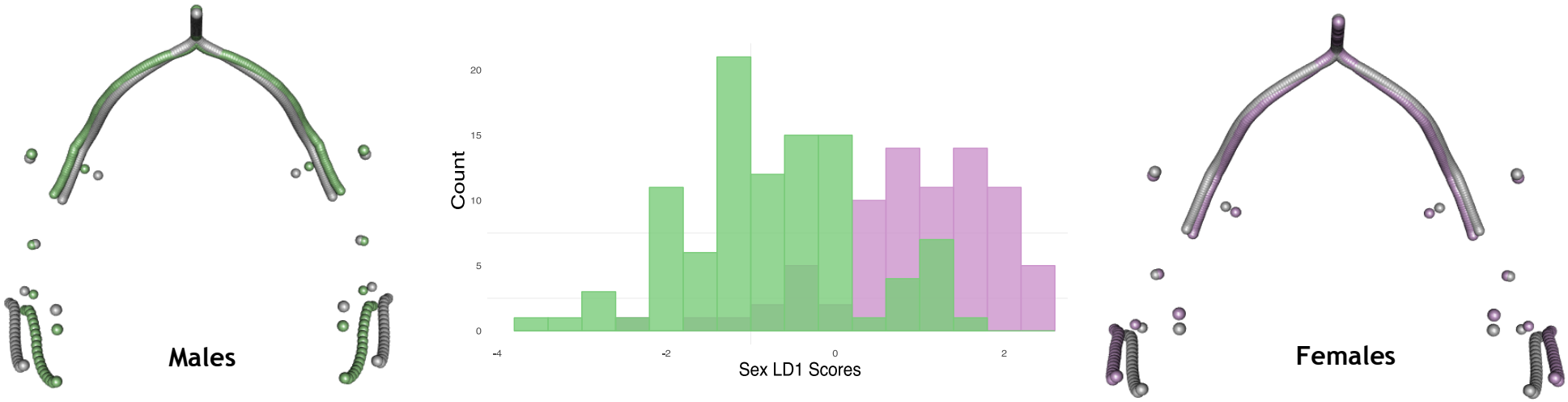

Figure 2—5: Sexual dimorphism in mandible shape as identified by a discriminant function analysis from the dorsal view and relative to a common consensus configuration of landmarks (grey landmarks). The left panel represents shape variation in males (green landmarks, and bars within the frequency histogram) whereas the right panel represents females (purple landmarks, and bars in the frequency histogram). Shape differences from the consensus have been magnified by a factor of 5 to enhance interpretation.

2.4.2 Quantitative Trait Loci (QTL) and Identification of Candidate Genes

Both the PC1 and PC2 from the MQM models had a QTL on LG18, while a QTL was present on LG7 for the PC2 model but not PC1. The effect size for the QTL on LG7 was higher (10.7%) than the QTLs on LG18 (6.7% for both models) (**Table 2-2; Figure 2-6)**. For the multivariate approach a QTL was also found on LG18 using the first two PCs both with and without sex as a covariate. The multivariate model for four PCs indicated a QTL on LG18 (with an effect size of 0.23%) when sex was a covariate. A QTL was found on LG10.2 for both of the four PC models. The multivariate models including ten PCs, had an additional 5 QTL unique from other models except for a QTL on LG7. The latter QTL was however present with and without sex as a covariate. In addition, the ten PCs model indicated an additional QTL on LG16 when sex was a covariate.

For LG18, Fst values were greater than 0.9 in 9 of the 14 genetic markers within the confidence intervals indicating selection is likely occurring in this region (**Figure 2-7**). The confidence interval was used as a starting point for investigations, and *zeb1* (located at position 6.75 cM on the genetic map on LG18 with an Fst value of 0.95) was identified as potential candidate gene for mandible shape variation. From the DFA at both of the nearest markers to *zeb1*, the mandible shape at the negative end of the LD1 axis, representing the AA genotype (LF in this cross) was wider than for the BB genotype (TRC in this cross) reflecting the shape differences that would be expected at the species level (**Figure 2-8**).

Table 2-2: QTL mapping results from the multivariate and MQM tests. For the multivariate models, all tests were run with and without sex as a covariate. Across models, LG18 and LG7 have the most QTL relating to mandible shape. Genome locations and 95% confidence intervals are included for each QTL. (LG = Linkage group; Pos = Position on the linkage group; LOD = Logarithm of the odds).

Model	Method	LG	Pos	LOD Score	Interval (cM)	Nearest Marker	Effect sizes
PC1	MQM	18	0	3.15	0 – 15	109.46112	7.9%
PC2	MQM	7	5	5.15	0 -15	193.987462	12.6%
		18	10	3.18	0 -19	41.248320	8.0%
		21	0	2.94	0 -17	210.488716	7.4%
PC1 & PC2	Multivariate	18	1.54	4.30	0 – 14	215.432511	0.2%/0.02%
PC1 & PC2 with sex as a covariate	Multivariate	18	1.54	4.59	0 - 16	215.432511	0.06%/0
PC1 – PC4	Multivariate	7	28	5.72	2 -34	21.2195347	1.8%/0.27%
		10.2	1.44	4.73	0 – 10	94.1628475	0.65%/1.52%
PC1 – PC4 with sex as a covariate	Multivariate	10.2	1.44	4.47	0 -11	94.1628475	0.18%/0.46%
		18	1.54	3.85	0 - 21	215.432511	0.23%/0
PC1 – PC10	Multivariate	1	30.85	5.06	14 -39	7.4421711	0.21%/0.001%
		5	60	4.34	56 – 70	10.6870455	0.03%/0.94%
		6	47	4.66	23 – 50	20.1015081	0.02%/0.42%
		7	5	6.39	1 – 33	193.987462	0.31%/0.03%
		17	17	4.87	9-64	154.63018	0.01%/0.61%
PC1 – PC10 with sex as a covariate	Multivariate	1	30.85	4.80	12 – 46	7.4421711	0.25%/0.08%
		5	60	4.36	56 – 71	10.6870455	3.2%/1.5%
		6	47	4.57	24 – 50	20.1015081	0.18%/0.83%
		7	5	4.39	0 -34	193.987462	0.61%/0.13%
		16	38	3.84	35 – 57	42.1183857	0.46%/0.59%
		17	42	4.90	9 - 62	154.63018	0.77%/0%

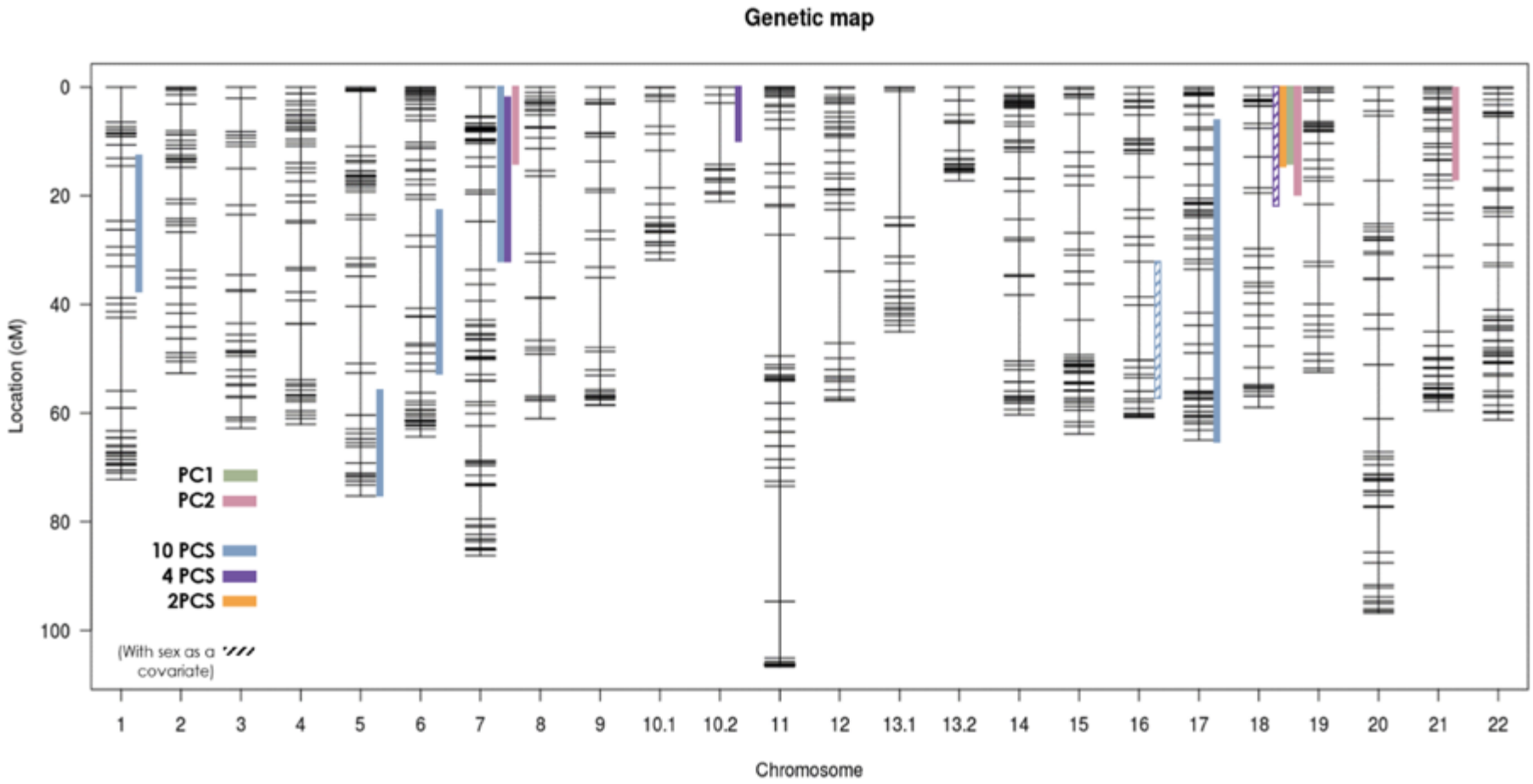

Figure 2—6: The genetic map for the F2 hybrid population (n = 176) with 95% confidence intervals for QTL derived from the multivariate and MQM models for mandible shape. For the multivariate models, all tests were run with and without sex as a covariate. The coloured lines associated with each model represent the 95% confidence intervals for each QTL. LG18 (0-21cM) had the most QTL across models relating to mandible shape.

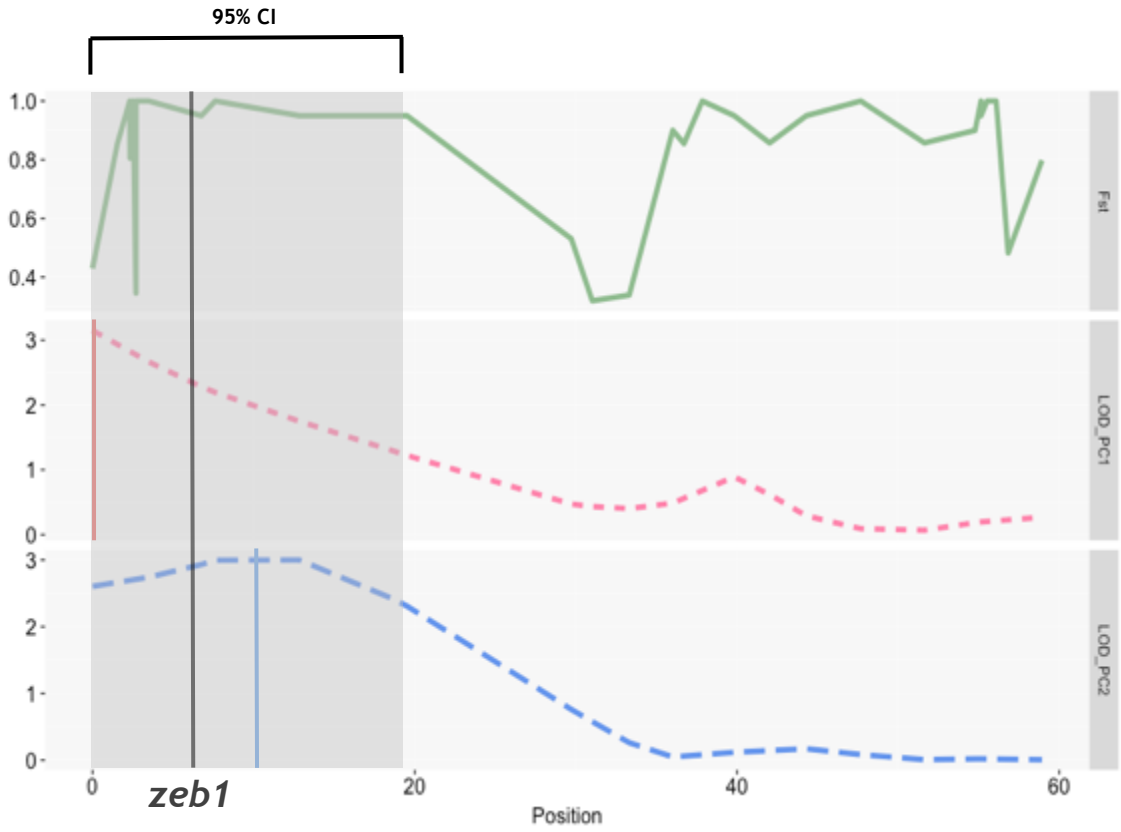

Figure 2—7: The top panel provides a line plot of Fst values (green) while the middle and bottom panels provide LOD scores on LG18 for PC1 (red) and PC2 (blue) MQM models respectively. The QTL confidence intervals are represented by the dark grey box for comparison of the LOD scores for the QTL with population genomic trends. The vertical line across panels indicates the location of the nearest marker to the highest LOD score within the QTL confidence intervals on LG18. Notably, Fst values reach an extended peak within the QTL region.

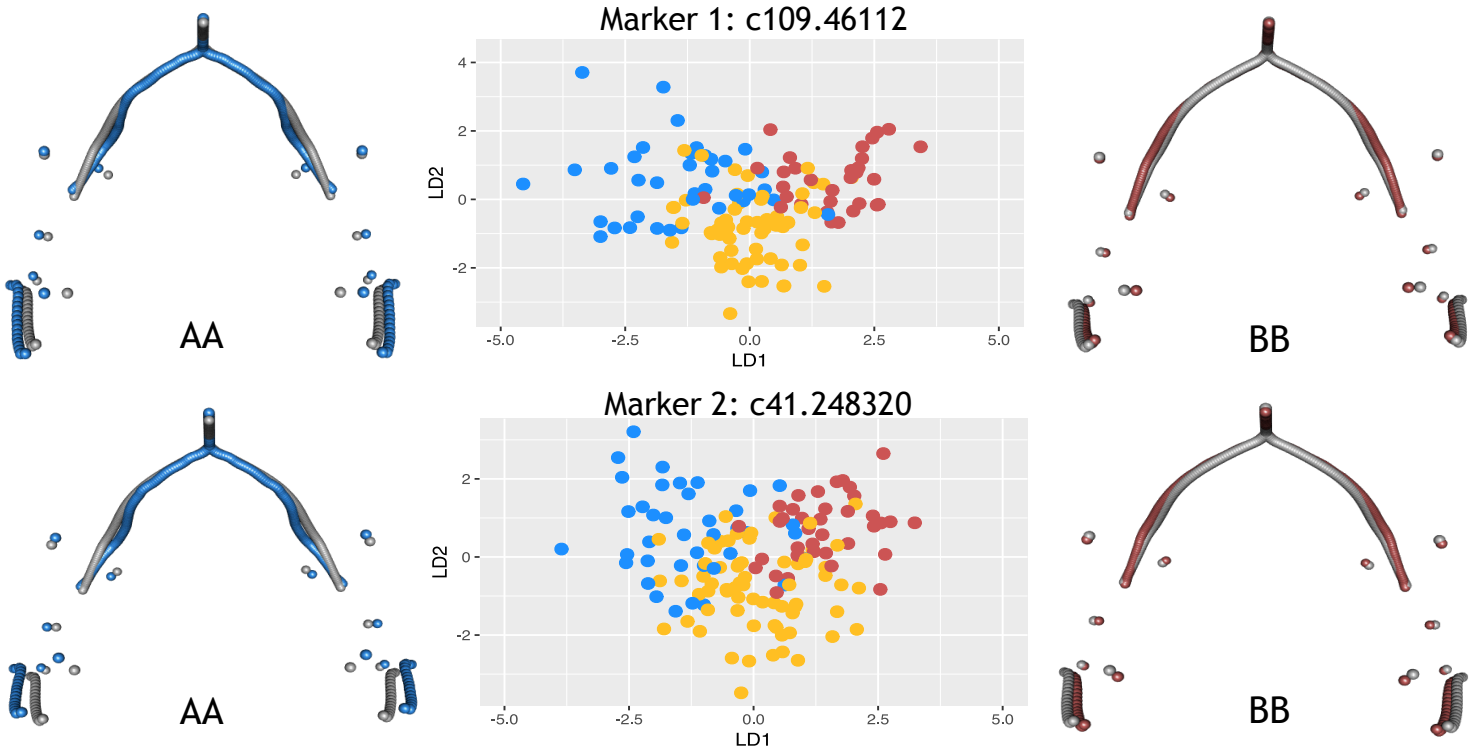

Figure 2—8: Scatterplots showing the classification of mandible shape based on genotypes from two markers flanking peak LOD scores for QTL (AA in blue, AB in yellow, and BB in red). Classification was derived from the LD1 and LD2 scores from a discriminant function analysis for each marker. Each row of panels provides a depiction of shape change by modelling landmark variation along the LD1 identified for each discriminant function analysis. In this cross, an AA genotype is LF and BB is TRC. The top panels represent the shape for and AA and BB genotype associated with marker c109.46112 (identified from the PC1 MQM model) for expected shape with an AA or BB genotypes while the lower panels represent shape changes associated with marker c41.248320 (identified from the PC2 MQM model); for both, the nearest candidate gene was *zeb1*. The consensus form is shown in grey and the modelled shape differences were magnified by a factor of 3 to enhance interpretation.

2.4.3 Investigating Candidate Gene Expression

Both *bmp4* and *zeb1* were expressed all over the craniofacial region in TRC and LF, however, there was no clear band of expression of *zeb1* or *bmp4* in the mandible in either species. *Col1a1* was primarily expressed in the developing bone in the pectoral fin and in the spine, with some expression visible in the mandible and craniofacial region (**Figure 2-9**). Control embryos for both species showed no evidence of gene expression.

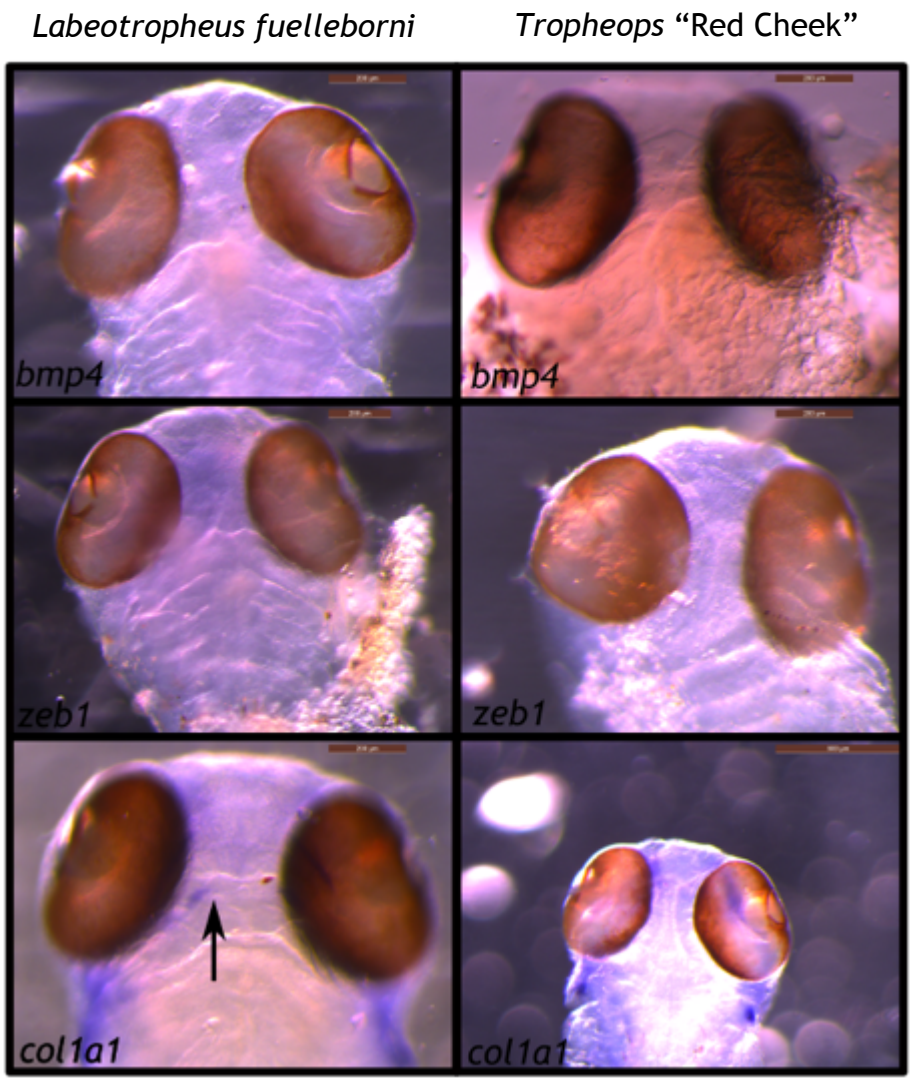

Figure 2—9: Expression patterns of the *bmp4*, *zeb1* and *col1a1* with a focus on the mandible on *Labeotropheus fuelleborni* (LF) and *Tropheops* "Red Cheek" (TRC) and embryos at stage 16 (4-5 days post fertilisation). The sample size was approximately 25 TRC from three different broods and 20 for LF from two different broods, divided between each probe and the control. The black arrow indicates expression in the mandible.

2.5 Discussion

Understanding the genetic architecture of phenotypic traits can lead to a deep understanding of how adaptive variation is generated (Hendrikse, Parsons and Hallgrimsson, 2007; Irschick *et al*. 2013). I aimed to address the genetic basis of the mandible in a high degree of detail by taking into account the contribution of different mechanisms of divergence (both species and sex) and implementing new methodology to enhance existing knowledge of craniofacial variation in adaptive radiations. The major axis of variation in mandible shape (PC1) was not affected by sex and with its effects on shape mostly related to mandible width, it likely reflecting shape variation between the parental species. This suggests sex-based variation is not driving the main pattern of adaptive divergence in cichlid mandibles.

The major difference in mandible shape between TRC and LF was width with my QTL findings suggesting that genetic mechanisms in several genomic regions play a role in determining variation for this trait. Specifically, the identification of *zeb1* as a candidate gene provides supporting evidence that it contributes toward mandible width (**Figure 2-8**). Although not previously investigated in cichlids, *zeb1* is known to be is associated with neural crest derived tissues, this includes the craniofacial region, and epithelial mesenchyme transition, which is crucial for the emergence of morphological structures and other key processes such as palate formation (Thiery *et al*. 2009; Shin *et al*. 2012; Zhang, Sun and Ma, 2015). Furthermore, mice with a *zeb1* mutation exhibit craniofacial abnormalities including secondary cleft palate (Takagi *et al*. 1998; Shin *et al*. 2012).

Key to the cichlid radiation is the presence of sexually dimorphic colour patterns, and previously, *zeb1* has also been implicated as a potential regulator of pigmentation in cichlids (Kocher, 2004; Albertson *et al*. 2014). Pigmentation is well known to be determined by NCCs, therefore, mutations in *zeb1* could have pleiotropic effects on both sexually dimorphic craniofacial morphology and pigmentation in cichlids. During key periods of craniofacial development, differences in the congregation of NCCs differs between cichlid species with differing jaw morphologies, but such approaches have not yet been extended to

periods of pigmentation development (Albertson and Kocher, 2006). Future avenues for research could look at the role of NCCs in sexually dimorphic pigment development and craniofacial shape and establish whether they are linked through a common developmental mechanism. If they are linked, this could highlight the importance of sexual dimorphism to the process of the African cichlid radiations.

Alongside craniofacial variation, cichlids are diverse in tooth phenotypes with widely spaced unicuspids in plankton-feeders to tightly packed tricuspids in algal scrapers (Albertson, Streelman and Kocher, 2003b; Streelman *et al*. 2003). Such phenotypes could also be linked to variation in *zeb1* through its pathway interactions. Interestingly, in mice, *zeb1* has been implicated in tooth development and is mediated by *bmp4* (Shin *et al*. 2012). *Bmp4* is posited as a key factor in craniofacial divergence in adaptive radiation; higher *bmp4* expression during development is associated with a biting morphology in both Darwin's finches (Abzhanov *et al*. 2004), and African cichlids (Albertson *et al*. 2005; Albertson and Kocher, 2006). Indeed, *bmp4* has been implicated in the control of cichlid dentition phenotypes (Streelman *et al*. 2003; Fraser, Bloomquist and Streelman, 2013). As the genomic location immediately surrounding *zeb1* appeared to be under strong selection, it is reasonable to suggest that *zeb1* is a candidate gene for adaptive divergence in the mandible. However, specific expression of *zeb1* or *bmp4* was not observed in the mandible of TRC or LF at stage 16. I would predict a difference in *zeb1* expression between the two species because of the difference in mandible width and it appears from the QTL that *zeb1* contributes towards jaw width (**Figure 2-8**). Nonetheless, if no expression in the mandible of either species exists at this stage it is possible that *zeb1* could still be important. A logical next step would be to assess multiple developmental stages where mandible development and differentiation occurs. Given that *zeb1* plays a role in NCC processes, it is possible that *zeb1* expression could be apparent at earlier stages than I examined here. Furthermore, given that the *bmp4* probe did not work as reliably as expected (Albertson *et al*. 2005), there could be an issue with the probe design conducted here.

Although smaller in magnitude compared to species differences, sex differences look to be following a trend that would aid in either a benthic or limnetic foraging strategy (Albertson *et al*. 2005). Specifically, female mandibles were narrower across the width of the jaw (from one side of the mandibular lateral line foramina to the other) and had more of "V" shape than males. Such shape variation would be expected in limnetic feeders, while the wider, more "U" shaped male mandibles would be expected to be found in benthic feeders. However, the female mandibles were wider than the males between the articular web on either side of the mandible. This could be due to hybridization or modularity of allelic effects on the cichlid mandible (Parsons, Marquez and Albertson, 2012). Furthermore, the female in the initial parental cross was an LF therefore this trend could reflect variation inherited from the original cross. How shape variation in the mandible might influence function and ability to handle external forces during feeding will be the subject of **Chapter 3**.

The type of sexual dimorphism in shape I identified is likely to be ecologically relevant and align with the ideas of ESD. However, it is unclear what ESD represents within the African cichlid radiations. It could be that ESD is a form of adaptive nested variation within the Malawi radiation. In a previous study on this same F2 cross, Parsons *et al*. (2015) suggested ESD in the lateral aspects of craniofacial shape with males possessing a steeper craniofacial profile that would like be more adept for biting relative to females. Similarly, sex-based differences in mandible shape have been reported for TRC and *Maylandia zebra* (MZ), with females possessing a longer, thinner jaw in comparison to males (McWhinnie and Parsons, 2019). In both cases these sex-based differences did not account for the majority of the variation supporting the idea that ESD is nested within broader patterns of divergence (Foster, Scott and Cresko, 1998; Riopel, Robinson and Parsons, 2008; Parsons *et al*. 2015).

Such sexual dimorphism could suggest an issue of genomic conflict involved with the determination of mandible shape. Further evolution of sexual dimorphism in the mandible could be limited by genomic conflict hence why it would represent smaller, more subtle divergence than what is occurring between species. For sexual dimorphism to evolve, especially for functionally important variation, there has likely been selection in favour of resolutions to genomic conflict (Cox

and Calsbeek, 2009). In some of the multivariate QTL models, the results differed when sex was included as a covariate and when it was not; a QTL on LG18 appeared for two multivariate models and a QTL on LG16 was present from the ten PC model (**Table 2-2**). Adding sex as a covariate limits the effect of sex on the model and therefore the QTL that appear under these conditions could represent areas under genomic conflict and the effect of these regions are perhaps covered by conflicting sex effects. Furthermore, the data suggests that some QTL for mandible shape could be linked to the main sex determining region in cichlids (Cox and Calsbeek, 2009; Parnell and Streelman, 2013). The sex determining region appears to be frequently involved with the genetic basis of mandible shape (LG7 appearing in 75% of all models); this even included findings of QTL from models that included sex as a covariate. The need to resolve genomic conflict arising from sexually antagonistic selection on mandible shape could be why LG7 frequently appears in QTL models. Linkage to the sex determining region could help resolve sexual conflict and allow selection for sexually dimorphic craniofacial traits as these loci could co-segregate together more frequently and allow for the evolution of sexual dimorphism.

Although not investigated here, it is possible that there are important candidate genes for craniofacial and mandible divergence in the sex determining region. Indeed, in Lake Malawi cichlids (including LF) sexual conflict in colouration exists whereby females have a characteristic “orange blotch” (OB) phenotype which acts as camouflage against the background of the rocky habitat (Roberts, Ser and Kocher, 2009). Sexual selection plays a key role in the Lake Malawi radiation and this relies on male colour patterns; in contrast to females, the OB phenotype is therefore predicted to confer a negative effect on male fitness (Van Oppen *et al*. 1998; Roberts, Ser and Kocher, 2009). The resolution of this sexual conflict is in the form of tight linkage of the locus responsible for the OB phenotype (*Pax7*) with a sex determining region on LG5 (Roberts, Ser and Kocher, 2009). Therefore, the prevalence of LG7 in the QTL models presented here could represent a resolution to the sexual conflict resulting from divergent selection pressures facing the sexes. Interestingly, LG7 also appears in other QTL studies on cichlid craniofacial variation suggesting a key role for sexual dimorphism in adaptive craniofacial and mandible divergence (**teeth shape**: Albertson,

Streelman and Kocher, 2003a; **mandible mechanical advantage**: Albertson *et al*. 2005; **maxilla bone architecture**: Albertson, Cooper and Mann, 2012; **craniofacial shape**: Parsons *et al*. 2015; and **snout size**: Conith *et al*. 2018).

2.6 Conclusions

Most radiations are characterised by rapid and diverse evolution of trophic morphology as this determines diet and the ability to process food. Therefore, understanding the molecular basis of their evolution can shed light on the process of adaptive radiation. African cichlids display an extensive range of variation in craniofacial shape and studying the genetic basis of this is key to understanding how key vertebrate innovations, such as the mandible, have evolved. This chapter presents a new candidate gene not previously characterised for the mandible, *zeb1*. As well as looking at divergence between species within a radiation, I also assessed the contribution of sexual dimorphism in mandible shape. There were fine-scale, ecologically relevant differences in mandible shape between the sexes suggesting that ESD is an additional source of divergence. Furthermore, the prevalence of LG7, the sex-determining region in this cross, in the QTL models suggests a linkage to this region as a resolution to the sexual conflict. In addition, as QTL models which had sex as a covariate revealed additional QTL, this suggests that the sexual conflict has not been fully resolved. It is likely then that the Lake Malawi cichlid radiation is therefore comprised of the main axis of divergence between biting and suction feeders followed by smaller, nested levels of variation within ecological niches between species and sexes; this could explain why this radiation has been so successful.

Chapter 3: Interspecific and Sexually Dimorphic Functional Divergence in African Cichlid Mandibles

3.1 Abstract

For vertebrates, the mandible often provides a direct link with prey during foraging making understanding this trait especially important. The mandible has most often been examined at the interspecific level but to deepen our understanding of why divergence occurs it should be a priority to examine intermediate phenotypes as well as other levels of variation. Therefore, this chapter explores functional variation in the mandible from three different perspectives: 1) interspecific divergence using *Tropheops* "Red Cheek" (TRC), and *Labeotropheus fuelleborni* (LF), two species that are members of the Malawi cichlid radiation, 2) at the level of hybrids between TRC and LF which possess intermediate phenotypes, and finally 3) at the level of sexual dimorphism. For each level, finite element analysis (FEA) was used to assess function through the modelled ability to handle external loading likely to be experienced during feeding. Loading was placed across different widths of the mandible to simulate how each species would encounter external forces during their respective feeding modes, plucking and scraping. The FEA highlighted different structural adaptations in TRC and LF which aid in dissipating stress during feeding. Furthermore, males possessed a wider mandible than females and appeared to be better at handling loading suggesting that they possess an advantage for a biting mode of feeding. The hybrids showed high levels of stress across the mandible, but the female showed less stress than male. This suggests a wide range of variation in the ability to handle loading in the hybrids. Together, these results suggest that interspecific divergence is maintained by functional advantages that are favoured by divergent selection and that divergence between sexes is ecologically relevant.

3.2 Introduction

The relationship between form, function and ecology reflects how an organism utilises its environment and is a key component adaptive divergence (Wainwright, 1996; Schluter, 2000). Related to this is the process of adaptive radiation which results in a multitude of species which have diverged from a common ancestor and are adapted for different ecological niches (Schluter, 2000). Indeed, this process is thought to occur as a result of competition for food and habitat that then drives selection and explosive divergence (Schluter and McPhail, 1993; Skulason and Smith, 1995). As a result, divergence in trophic morphology has been suggested as key to the adaptive radiation process as it represents a means for direct interaction between an organism and the prey resources of an environment (Streelman and Danley, 2003).

For teleosts, adaptive divergence has occurred frequently along a benthic/limnetic habitat axis. This has resulted in similar patterns of morphological change across lineages that relate to the biomechanics of feeding performance (Albertson *et al*. 2005; Skulason et al. 2019). The feeding efficiency of an organism is in part limited by the shape of trophic morphology as this places constraints on the shape and size of prey that can be consumed and the speed or strength of the movements used for capture (Wainwright and Richard, 1995). A clear example is found in sunfish whereby pumpkinseeds (*Lepomis gibbosus*) feed on hard-shelled snails and in turn possess stronger and larger pharyngeal jaws and associated muscles in comparison to the bluegill (*Lepomis macrochirus*), which feeds on zooplankton (Mittelbach, 1984; Mittelbach, Osenberg and Wainwright, 1992; Wainwright, 1996). While this example highlights interspecific differences, understanding how functionally relevant morphology and biomechanics can vary at other levels of biological variation form an important component of adaptive divergence research (Wainwright, 1994; Cooper *et al*. 2010).

Specifically, divergence is most often studied between species while divergence within populations in the form of polymorphisms or ecomorphs is seen as a step toward speciation. However, relatively few studies of functionally relevant differences between sexes have been conducted. This may miss a crucial source

of variation for adaptive divergence. Sexual dimorphism is often considered only within the context of secondary sexual characters but there is an idea that adaptive differences can occur between sexes (Shine, 1989). This phenomenon is known as ecological sexual dimorphism (ESD) and how it contributes to broader patterns of divergence has only rarely been considered empirically. Nonetheless, sex differences in trophic morphology have been documented for some taxa and suggest a link to resource use (Selander, 1972; Shine, 1989; Temeles and Roberts, 1993; Temeles, Miller and Rifkin, 2010).

So far examples of ESD (discussed in detail in **Chapter 1**) have a limited demonstration of functional variation. In the threespine stickleback (*Gasterosteus aculeatus*), sexual dimorphism in trophic morphology is evident with jaw protrusion, a functionally relevant trait in the context of feeding performance, differing between sexes (McGee and Wainwright, 2013). Notably, sticklebacks possess sexual dimorphism in parental care with males building nests with their mouths, while females would seem to only need to commit their mouths to foraging. Thus, interactions between functions could alter both the shape and biomechanics of trophic structures. Mouthbrooding could provide an important influence on how ESD unfolds in the trophic morphology of other fishes such as reef cardinalfish (family Apogonidae) and cichlids (Barnett and Bellwood, 2005; tkint *et al*. 2012).

During feeding, trophic morphology can be subject to mechanical loading which causes bone to remodel itself. This can enhance adaptation with the bone-specific response known as “Wolff’s Law” (Wolff 1892; Owen *et al*. 2012). Specifically, with increased mechanical loading, more bone is deposited by osteoblasts while decreased loading causes reabsorption of bone through the action of osteoclast cells (Witten and Huysseune, 2009). Such load-induced dynamics can create a constant cycle in addition to normal bone turnover that allows bone to change shape and structure in response to different mechanical loading regimes (Lanyon *et al*. 1982; Owen *et al*. 2012). With different foraging modes and associated craniofacial morphologies it is likely that the mechanical loading of specific bones will vary between species and possibly sexes. Indeed, a biting mode of feeding is likely to result in craniofacial bones being subjected to overall higher levels of mechanical loading likely leading to an increase in bone

deposition relative to a suction mode of feeding (Parsons *et al*. 2014). Bone is also fundamentally shaped by mechanical loads imposed by basic functions during development. For example, Hu and Albertson (2017) reported that increased length of the retroarticular process of the mandible was associated with high levels of gaping behaviour in African cichlid larvae. Therefore, exploring how bone responds to mechanical stress can shed light on how it can influence adaptive responses to the environment.

For trophic morphology the mandible forms a direct link between the organism and its environment. Indeed, the shape of the jaw is a useful predictor of function, feeding ecology, and performance in fishes (Westneat, 1995; Wainwright *et al*. 2004; Albertson *et al*. 2005). The mandible can directly limit the prey shapes and sizes that can be eaten as well as the power and rate of movements that are required to capture prey found in the water column (Westneat, 1995). There are general mandible morphologies that are associated with evolution along the benthic/limnetic habitat axis. Short, wide jaws facilitate biting, whereas suction feeding benefits from long, thin gracile jaws (Albertson *et al*. 2005). Furthermore, these shapes relate directly to the mechanical advantage of the mandible; this is calculated for both opening and closing using a ratio between the lengths of the in-levers (closing or opening) and the out-lever of the mandible (e.g. Wainwright and Richard, 1995; Westneat, 1995; Wainwright *et al*. 2004; Albertson *et al*. 2005). A higher mechanical advantage results in strong but slow movements for biting (often used for benthic feeding), conversely, suction feeding requires a low mechanical advantage for rapid but delicate movements (often used for limnetic feeding) (Albertson *et al*. 2005). Our understanding of the function of the mandible from its outward anatomical variation between species provides a strong basis for deeper investigation of this complex structure.

Finite element analysis (FEA) is a tool that is proving to be useful for assessing the performance of complex structures under mechanical load and for assessing links between form and function (Ross, 2005; Polly *et al*. 2016). FEA can be used as a tool in evolutionary biology to assess how anatomical structures cope with loading and address a range of different research questions (Polly *et al*. 2016). Various different ecological scenarios can be tested with FEA such as testing the

ability to resist bites from predators (e.g. Rivera and Stayton (2011)), biting in different ways (e.g. Dumont, Piccirillo and Grosse, 2005; Pierce, Angielczyk and Rayfield, 2009) and structural adaptations for feeding (e.g. Hulsey *et al*. (2008)). Research has moved towards building a synthesis between morphometrics and FEA as this could be a powerful way to investigate form and function relationships at different levels within an evolutionary context (Polly *et al*. 2016).

With extensive and well-documented variation in craniofacial and mandible shape (e.g. Albertson and Kocher, 2001; Cooper *et al*. 2010; Powder *et al*. 2015), African cichlids are an excellent model with which to explore functional divergence using FEA. Furthermore, by combining FEA with investigations of morphological differences between the sexes, this can provide insights into whether sexual dimorphism is likely to be ecologically relevant. Previous cichlid research has shown that males possess steeper facial profiles relative to females, and in line with a morphology associated with a biting mode of feeding (Parsons *et al*. 2015). However, as in other cases, confirmation of ESD was difficult as there are no criteria which are reliable in practice and other factors may be involved in the evolution of sexually dimorphic trophic structures (Shine, 1989). However, given the link between the shape of trophic morphology and its ecology, testing for sexual dimorphism in functional performance in the high level of resolution that FEA allows could potentially strengthen a case for ESD.

Additionally, African cichlids are also an excellent model with which to test for performance trade-offs between phenotypes. Their recent divergence and shared genetic background allow for cichlids to be hybridised in the laboratory to create phenotypes that are intermediate between parental species. Such hybrid phenotypes are generally predicted to have a lower fitness which supports the idea of disruptive selection (Mayr 1963; Grant and Grant, 1992; Hatfield and Schluter, 2006). However, in some cases hybrids may actually achieve higher fitness and provide variation for further adaptive divergence (Arnold and Martin, 2010). Indeed, for African cichlids hybridisation is often posited as a facilitator of evolvability and rapid divergence (Seehausen, 2004; Albertson and Kocher, 2005; Parsons et al. 2011). Therefore, this chapter

provides an opportunity to assess the functional consequences of hybridisation within the context of interspecific divergence and adaptive radiation.

This chapter assesses functional variation of the mandible between species and the sexes in relation to the mandible shape. Rather than attempt to recreate *in vivo* loading, this study is a comparative analysis of the ability of the mandible to cope with external compressive loading that would likely be experienced during feeding by *Tropheops* "Red Cheek" (TRC) and *Labeotropheus fuelleborni* (LF). For the interspecific comparison I predict that as *Labeotropheus fuelleborni* which possesses a classic biting mode of feeding, will be superior at handling mechanical loading relative to the *Tropheops* "Red Cheek". With regards to sexual dimorphism, I predict that sexual dimorphism will be present in mandible shape and that FEA will show males to be superior at handling mechanical loading. Finally, for hybrids I predict that they will not out-perform either parental species in line with previous studies on adaptive divergence (Mayr 1963; Grant and Grant, 1992; Hatfield and Schluter, 2006).

3.3 Methods

3.3.1 Specimen Preparation and µ-CT Scanning

Both parental species are algal feeders but likely reduce competition through different foraging modes within the same ecological niche (Concannon and Albertson, 2015). Specifically, LF scrapes algae off of rocks whereas TRC plucks and 'nips' the algae off (Albertson, 2008; Albertson and Pauers, 2018). Within each species there are suggestions that there are sex differences in foraging locations as males are highly territorial (Ribbink *et al*. 1983). In addition, males use their mouths for biting and aggressive interactions include males locking their jaws together (Ribbink *et al*. 1983; Danley, 2011). Furthermore, as females are mouthbrooders, this could impose functional constraints on the mandible. To examine and quantify variation in mandible shape between LF and TRC, a 3D morphometric approach was conducted.

Firstly, 20 adult fish of a similar age and size were selected from aquarium populations held at the University of Glasgow and sacrificed with an overdose of

benzocaine solution following Home Office guidelines; fish were then frozen at -20°C until dissections took place. In line with ASPA animal welfare regulations, to reduce the number of animals euthanised, some of the fish selected had died of natural causes. The sample size comprised of approximately equal numbers of each sex confirmed by colouration, internal dissection of gonads and vent size (Moore and Roberts, 2017). To remove the mandible, it was disarticulated from the maxilla and the quadrate of the skull and then carefully dissected out. Mandibles were stored in 1X PBS to maintain hydration during the scanning process. As in **Chapter 2**, µ-CT scanning was conducted using a Bruker Skyscanner 1172 (Bruker, Billerica MA). The µ-CT scanning parameters remained constant at 70kV and 10 µm resolution using the largest camera for all mandibles. Processing of the raw images was carried out using NRecon (version 1.6.9.18) to ensure alignment of each µ-CT image slice. Settings, including 'smoothing', 'ring-artefacts reduction', and 'misalignment correction' remained constant across specimens. One TRC model was removed from the sample due to poor model quality. For TRC there were 9 specimens, (female = 4 and male =5), and for LF there were 10 specimens (female = 5 and male = 5).

3.3.2 3D Model Generation for Morphometrics

To create 3D models for shape analysis, ScanIP (Version 7.0; available at: https://www.simpleware.com/) was used on the processed images from above. For each sample, the stack of µ-CT image slices was loaded with a "Pixel Skip" value of 3 to reduce triangles on the model (reducing file size from ~1GB to 50-100MB per specimen). To reduce noise and smooth the model surface, the "Recursive Gaussian" filter was used with settings adjusted an individual basis depending on need. Each model was then segmented using the "Interactive Threshold" function to highlight regions of bone based on greyscale values from the scanning process, followed by the "Flood Fill" function which created a mask that included only connected areas of the model to remove scanning artefacts. The final steps of model creation involved smoothing the surface topology and reducing the number of triangles before exporting as an STL (stereolithography)

file. Before morphometrics could commence, the STL file was converted to a PLY file using MeshLab (available at: http://meshlab.sourceforge.net/).

3.3.3 Morphometrics and Analysis of the Parental Species

Mandible shape for both LF (n = 10) and TRC (n = 9) was quantified using 3D morphometrics; all analysis was conducted using R version 3.4.1 (R Core Team 2017) using the geomorph package (Adams and Otárola-Castillo, 2013; Adams, Collyer and Kaliontzopoulou, 2019) unless otherwise stated. Firstly, using Landmark Editor (available: http://graphics.idav.ucdavis.edu), 20 homologous landmarks reflecting functional relevance were manually placed on each model surface (e.g. Albertson and Kocher 2001 and Parsons *et al.* 2012) (**Figure 3-1**; **Figure 3-2**). Prior to analysis in R, landmark data in the form of pts files, were converted to a TPS file using Simple3D (IMP Software available: http://www.philadb.com/an-behav/imp/) (Zelditch, Swiderski and Sheets 2012a; Zelditch, Swiderski and Sheets 2012b). Shape analysis began with a Procrustes superimposition which rotated, translated, and scaled landmark data to a common size using the *gpagen* function. To ensure downstream analysis of shape only encompassed the bilaterally symmetric component of shape variation, asymmetry was also removed using the *bilat.symmetry* function (Klingenberg, 2015). Because the two different species had different allometric trajectories for mandible shape (tested by *proc.allometry*), an allometric correction was not conducted (Klingenberg, 2016).

To assess aspects of divergence between species and sexes in mandible shape, a series of steps were conducted. The effects of species, sex, and their interaction were assessed using a Procrustes ANOVA on landmark coordinates. Following this, to test *a priori* groupings of species and sex in the parental species, a discriminant function analysis was conducted using *lda* from the MASS package (Venables and Ripley 2002) on the first 5 principal components (90% of the variation). A principal component analysis (PCA) using *plotTangentSpace* was conducted on Procrustes coordinates to reduce the shape variables for the DFA. Generally, when the sample size is lower than the shape variables, the suggested

number of PCs to use is the number of samples divided by 4, which in this case is why 5 PCs were chosen as the shape variables for the DFA (Zelditch and Swiderski, 2018).

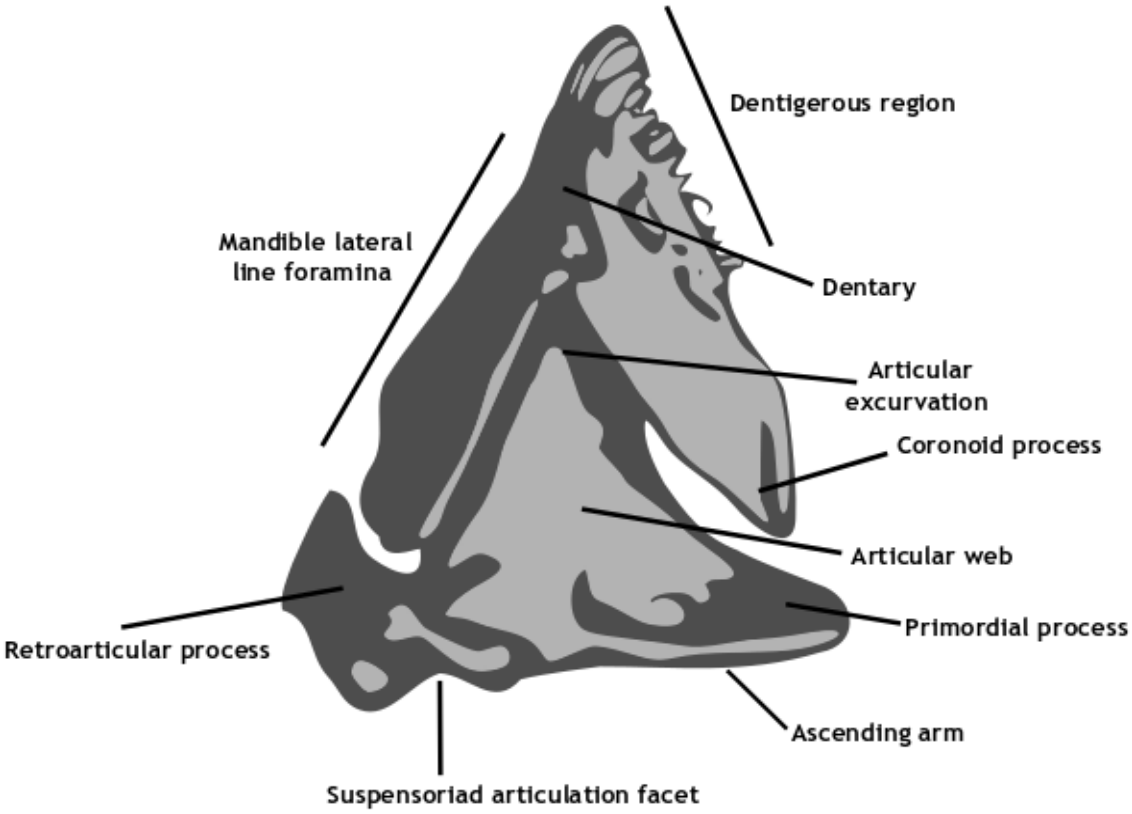

Figure 3—1: The labelled anatomy of the cichlid mandible from the lateral view based on Barel *et al*. (1977) and Parsons, Marquez and Albertson (2012).

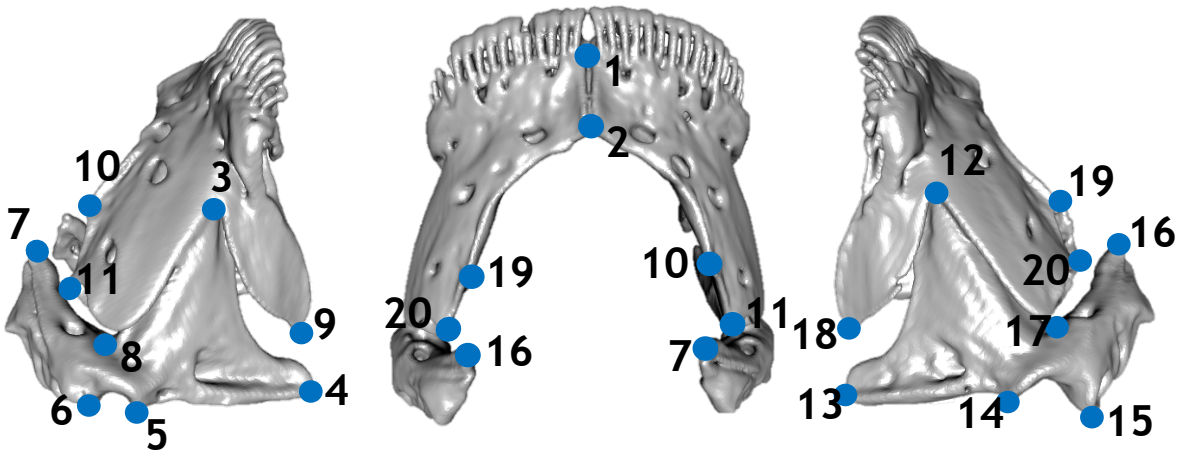

Figure 3—2: Landmarks used for morphometrics analysis. Each landmark represents a functionally relevant area of the mandible and selection was based on previous work on cichlid mandibles (Albertson and Kocher, 2001; Parsons, Marquez and Albertson, 2012). Landmarks represent the following anatomical locations: 1) dorsal tip of the midline; 2) ventral tip of the midline; 3) left lateral rostral tip of the articular excavation; 4) left lateral dorsal posterior tip of the primordial process; 5) left lateral dorsal edge of the lateral facet rim; 6) left lateral ventral edge of the lateral facet rim; 7) left lateral most rostral edge of the region ventral to the lateral line foramina; 8) left lateral ventral obturated foramen; 9) left lateral posterior tip of the coronoid process; 10) left lateral mandibular edge foramina; 11) left lateral ventral edge of the mandibular edge foramina; 12) right lateral rostral tip of the articular excavation; 13) right lateral dorsal posterior tip of the primordial process; 14) right lateral dorsal edge of the lateral facet rim; 15) right lateral ventral edge of the lateral facet rim; 16) right lateral most rostral edge of the region ventral to the lateral line foramina; 17) right lateral ventral obturated foramen; 18) right lateral posterior tip of the coronoid process; 19) right lateral mandibular edge foramina; 20) right lateral ventral edge of the mandibular edge foramina.

3.3.4 Specimen selection for Finite Element Analysis

To explore aspects of hybrid biology and sexual dimorphism for the finite element analysis, one F2 specimen of each sex of an intermediate shape between the two species were needed. F2 landmark data (for fully intact models) from **Chapter 2** (n=120) was appended to the TRC and LF landmark data from above; *gpagen* was used to conduct a Procrustes superimposition on the landmark data and *bilat.symmetry* used to extract the symmetrical component of the shape variation. To determine specimen position in shape space, a DFA was conducted using F2, LF and TRC as grouping variables. As before, *plotTangentSpace* was used to perform a PCA reduction and all resulting PC

scores were used for the DFA. A canonical variate plot was then created from this analysis based on the LD1 and LD2 scores to allow for a single male and female specimen to be selected from the middle of the F2 distribution (**Figure 3-3**). Two TRC and two LF specimens of each were selected for FEA based on the quality of the original model as not all would have been suitable.

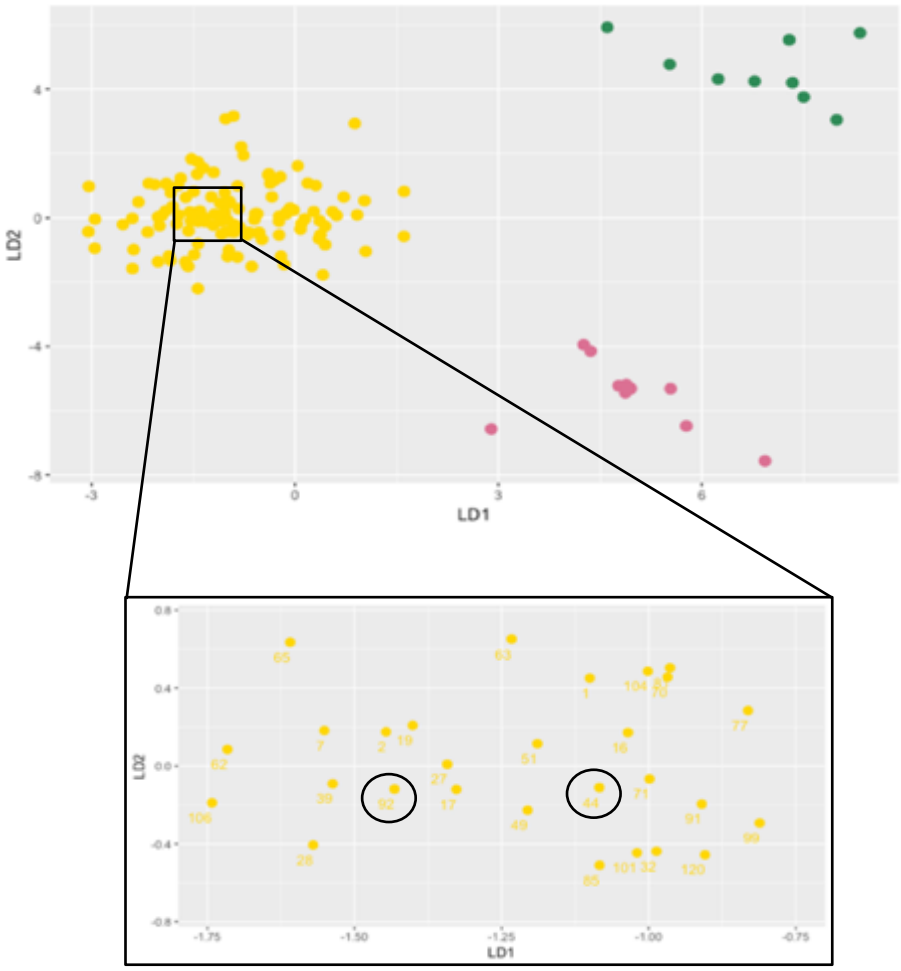

Figure 3—3: Scatterplot from a discriminant function analysis (DFA) on mandible shape used to aid specimen selection for the finite element analysis (FEA). *Tropheops* "Red Cheek" (TRC) is depicted in green, *Labeotropheus fuelleborni* (LF) in pink, and F2 hybrids are in yellow. The inset box indicates a magnified view of the F2 hybrid specimens; the two circles in the magnified box indicate the models which were selected for the FEA.

3.3.5 Mesh Creation for Finite Element Analysis

While the specimens used for FEA were identified from morphometrics, the models used for morphometrics were different to those used for the FEA. This was because there are a number of steps that need to be followed to produce a model specifically appropriate for a finite element model (Richmond *et al*. 2005; Ross, 2005; Panagiotopoulou, 2009; Peterson and Müller, 2018). Once specimens were selected, the meshes were created from the original geometry of the 3D model following a voxel-based approach using the software ScanIP (Version 7; available at: https://www.synopsys.com/simpleware.html). This method takes each voxel in the 3D model and converts it into a finite element mesh joined together at nodal points resulting in the generation of a mesh which accurately reflects the shape of the structure of interest (Rayfield, 2007; Panagiotopoulou, 2009). Tetrahedral elements were selected for each mesh generation over quadratic elements due to their lower computational demands; for biological structures there are minimal differences in results between the two element types (Dumont, Piccirillo and Grosse 2005). Completed meshes were checked visually and the mean in-out and edge length aspect ratios of elements calculated by the software were reviewed to ensure the elements were as uniform as possible (Stayton, 2009). The in-out aspect ratio and edge length aspect ratio relate to the shape of the individual elements that comprise an FEA mesh. To ensure a quality FEA mesh, elements should be as close to a 'perfect' triangle as possible; elements which are too elongated can result inaccurate results (Stayton, 2009). The documentation within the software recommends a value greater than 0.1 for the mean in-out aspect ratio and less than 10 for the mean edge-length ratio for a high quality FEA mesh.

Each element was then assigned material properties (Young's modulus and Poisson's ratio) which define how the mandible would behave under loading. The Young's modulus relates to the elasticity of the material and is the measure of the deformation of the material as a result of a given axial load, whereas the Poisson ratio is defined as a measure of the lateral strain divided by the axial strain which represents how the material will expand or contract to maintain volume (Richmond *et al*. 2005). The bones were assigned homogenous material properties to allow the focus to be on the shape of the mandible and for

simplicity as the bone density did not differ between species (also reported by Albertson, Cooper and Mann, 2012). For each element of a given mesh, the Young's modulus was assigned 6 GPa based on existing experimentally tested material property information for fish bone (Horton and Summers, 2009; Cohen *et al*. 2012) while a Poisson's ratio of 0.3 was assigned as this has been used previously in vertebrate FEA modelling of bone (Dumont, Piccirillo and Grosse, 2005; Hulsey *et al*. 2008). Although asymmetry was removed for morphometric analysis, it was retained for FEA models as it is a natural component of mandible development and likely to influence mechanical loading (see Stewart and Albertson (2010)).

3.3.6 Finite Element Analysis

To examine the ability of the mandible to cope with loading, each model was tested with compressive loading on the teeth. The focus was how this bone copes with compressive loading that could be experienced by the fish in their natural environment during scraping and plucking. Therefore, bite forces originating directly from the muscles attached to the mandible were excluded from this analysis. Using Abaqus CAE (available at: https://www.3ds.com/products-services/simulia/products/abaqus/abaquscae/), each mesh was assigned the same boundary conditions to prevent it from moving into space whilst also allowing for natural movement and loading conditions. The boundary constraints for each model were placed along each ascending arm of the mandible as this is where the adductor mandibulae, involved in jaw closing, attach. Multiple nodes were used for the boundary constraints as using a single node can result in unrealistically high stresses and strains in the surrounding area (Richmond *et al*. 2005). The left side of the mandible was constrained in all three directions (x, y and z) whereas the right side was constrained along y and z so as to allow realistic lateral movement for flexibility and deformation during loading (**Figure 3-4**). To reflect species differences, different loading scenarios were conducted for each mesh (**Figure 3-4**). The four loading scenarios represented the compressive stress a mandible would likely encounter during foraging in the two species. This included force being applied equally across four

different distances across the width of the mandible (approximately 25%, 50%, 75% and 100%). LFs likely use the full width (75%-100%) of the mandible during scraping and are substantially wider and straighter than TRC which are rounder and narrower and likely only use a small proportion of the width (25-50%) of the mandible when plucking.

Previous FEA modelling on the cichlid craniofacial apparatus has used a wide range of forces for loading scenarios. Cooper *et al*. (2011) tested bite force transmission through the skull and used 15N, which was suggested as an approximate value for bite force of an adult cichlid; Hulsey et al (2008) tested structural adaptations to molluscivory and used 1600 N, the maximum force a molariform can apparently withstand, and Peterson and Müller (2018) used between 0.02 to 0.5N to test bite force in developing embryos. After preliminary tests I elected to use 1N to simulate external, compressive loading on the mandible (adjusted for scale depending on the surface area of the mesh as described in the next section) as this produced stress values within the range that would be expected from FEA results in bone (the majority of the elements had stress values below 15MPa). As there is no existing data on bite force or loading on the mandible during feeding, values for loading in FEA have to be estimated. As this was a comparative analysis, the actual loading values are not necessary, with only the stress patterns being relevant (Rayfield, 2007). The results of the FEA analysis represents the stress, strain and deformation of each element of the mesh under the loading conditions (Richmond et al. 2005; Ross, 2005; Panagiotopoulou, 2009; Peterson and Müller, 2018). The von Mises stress is reported in this chapter as it is a reliable predictor of fracture in bone (Dumont, Grosse and Slater, 2009).

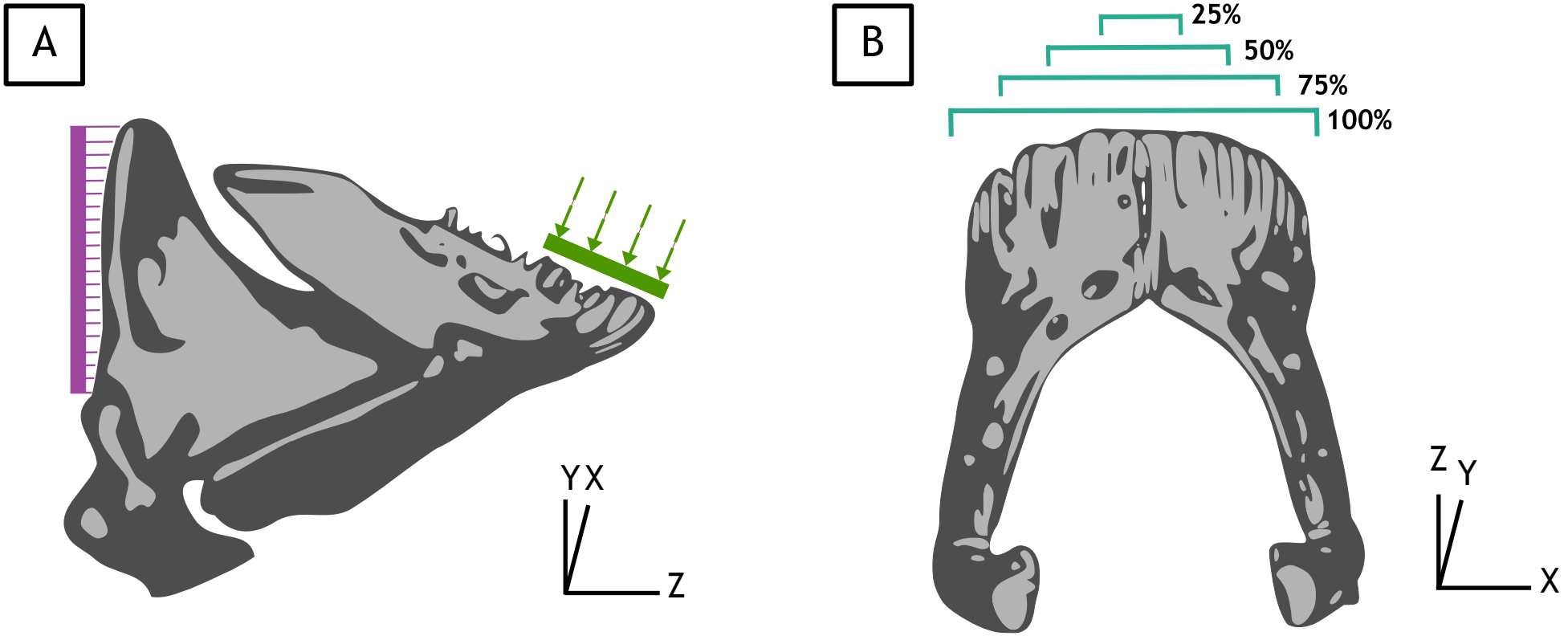

Figure 3—4: Graphical representations of the cichlid mandible from the lateral (A) and ventral (B) aspects. The lateral aspect (A) shows constraints on (shown in purple) the ascending arm for both sides of the model and the loading locations (in green). The ventral aspect (B) shows the location of modelled compressive forces (shown in green) and applied on to the teeth across four different, approximately symmetrical, spans across the mandible. The constraints anchor the mesh for the FEA; each model was constrained fully in all three directions on the left side and free to move along the X axis on the right side to allow for some natural flexibility. The orientation and coordinate system used is represented by the axes in the bottom right corner of each diagram.

3.3.7 Scaling of Mesh Loading

To accurately compare performance in response to force, the forces used were scaled according to the surface area of the mesh for each specimen selected for the FEA. This allowed any inferences about performance to be attributed to the shape of the structure rather than size related differences (Dumont, Grosse and Slater, 2009). However, although the optimal option would be to rescale each model to the same surface area, this can be both time-consuming and challenging. Instead, I followed the recommendations of Dumont, Grosse and Slater (2009) by ensuring that the applied force to surface area ratio was identical for each mesh. Therefore, the applied load from the model with the lowest surface area was used as a reference, and the force for each model was calculated using **Equation 3-1**. This resulted in each model having the same force-to-surface-area ratio to account for size differences between specimens. Surface area, rather than measures of model volume, were used as they are more accurate for scaling purposes when the structures in question differ in shape, as they do in this study (Dumont, Grosse and Slater, 2009).

$$F_B = (SA_B/SA_A) \times F_A$$

Equation 3-1: SA represents the surface area of both the reference (SA_A) and target (SA_B) models, F_A is the force used for the reference model, and F_B is the force to use for the target model.

3.4 Results

3.4.1 3D Morphometrics

Both species and sexes showed significant differences in mandible shape, however, there was no interaction between these factors which suggested that the level of sexual dimorphism was similar in both species (**Table 3-1**). From the Procrustes ANOVA, Species explained the greatest variation in mandible shape (57%) compared to sex (7%). *A priori* groupings of species were confirmed as the DFA (**Figure 3-5a**) showed a correct classification rate of 100% for both LF and TRC. Evidence for sexual dimorphism was strong in both species with the correct classification rate being 100% and 89% for males and females respectively (**Figure 3-5b)**. The major shape difference between groups was width, with LF mandibles being wider than TRC, and males having wider mandibles than females (**Figure 3-5**).

Table 3-1: The results of a Procrustes ANOVA conducted on shape coordinates to test for species and sex differences in mandible shape. P values were obtained through permutation procedures. Asterisks highlight statistically significant P values.

Factors	DF	Sum Sq	Mean Sq	R Sq	F	Z	P
Species	3	0.115890	0.115890	0.57340	26.1845	3.2590	**0.001*** **
Sex	3	0.014972	0.014972	0.07408	3.3828	2.3677	**0.016***
Species:Sex	3	0.004858	0.004858	0.02404	1.0977	0.6491	0.251
Residuals	9	0.202109	0.004426	0.32848			

***P <0.05, **P<0.01, ***P<0.001**

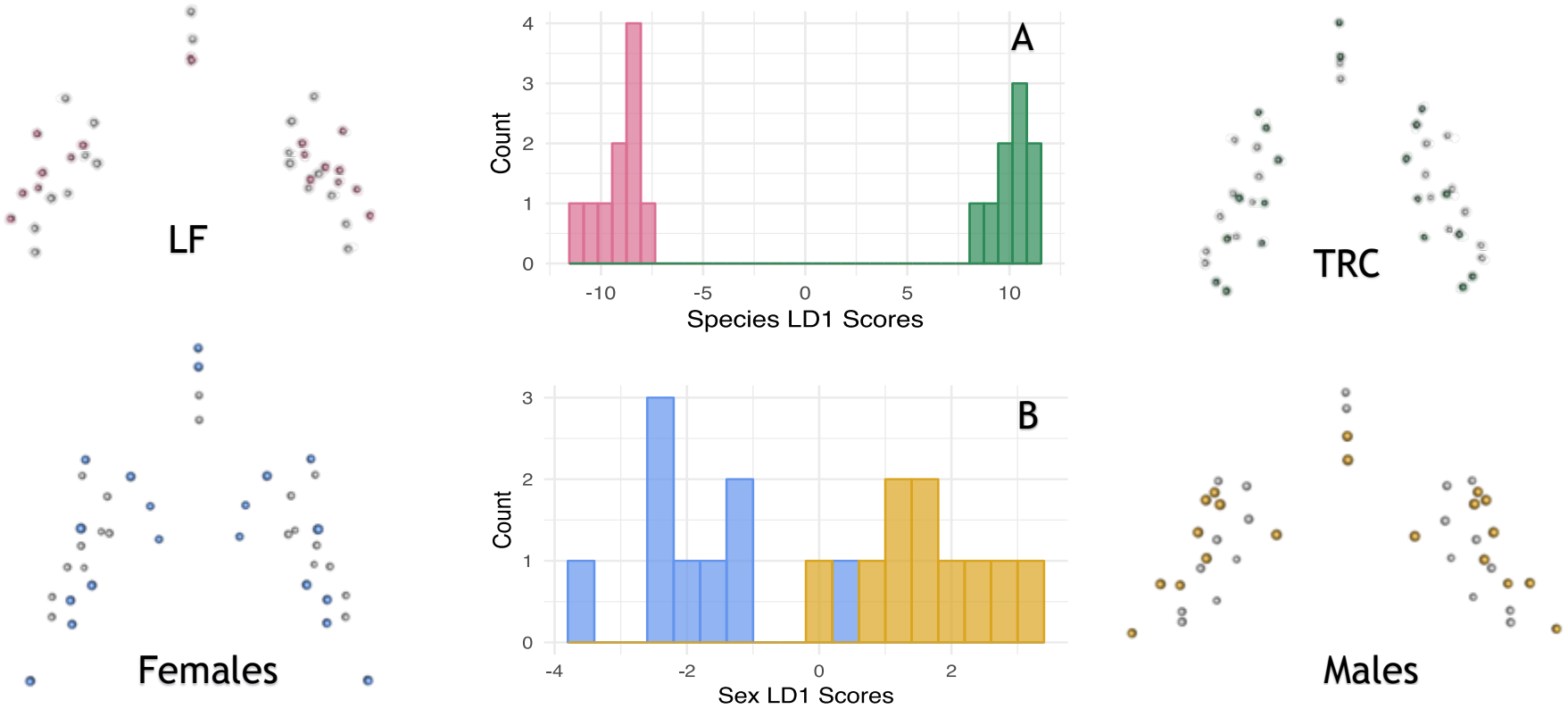

Figure 3—5: Frequency histograms for groupings derived from a discriminant function analysis using **A)** species with *Tropheop*s "Red Cheek (TRC) in green and *Labeotropheus fuelleborni* (LF) in pink and, **B)** sex with females in blue and males in yellow. Associated mandible shapes are shown either side of the frequency histogram with mean shape represented by the grey landmarks; differences have been magnified by a factor of 5 to aid interpretation.

3.4.2 Finite Element Analysis

All meshes had a similar mean aspect ratio and mean edge length ratio and the elements and meshes were of a high quality (**Table 3-2**).

Table 3-2: Mesh statistics for each specimen examined using Finite element analysis. Mesh loading and scaling is described in **Section 3.3.7**.

Model	Surface Area (mm^2)	Nodes	Mean In-Out Aspect Ratio	Mean Edge Length Aspect Ratio	Load (N)
LF Female	168	99,765	0.79	1.71	1.42
LF Male	402	289,588	0.80	1.68	3.44
TRC Female	185	87,173	0.76	1.79	1.57
TRC Male	171	82,359	0.78	1.72	1.45
F2 Female	140	81,878	0.79	1.72	1.19
F2 Male	118	62,529	0.75	1.83	1

With regards to the parental species models, when compared with LF, both TRC models had more areas of stress across the mandible when loading was applied across the entire width and approximately 75% of the width of the jaw to simulate how an LF would forage (**Figure 3-6** and **Figure 3-7**). Additionally, all four models showed stress laterally on the mandible at differing degrees, in particular at the edge of the articular web where the values of stress are 10 MPa and above, indicating fracture and failure would occur in this region. The LF models showed minimal stress across the dentary region of the mandible whereas the TRC models showed slightly more stress across this region when loaded in this way. The "wings" of the mandible in LF showed minimal stress when force was loaded across 100% and 75% of the width. This suggests LF possesses structural adaptations for dissipating load across the width of the mandible.

Placing load across 50% and 25% of the width of the jaw was intended to simulate how a TRC would come into contact with loading during feeding (**Figure**

3-8 and **Figure 3-9**). Under these conditions, the TRCs showed stress across the dentary region and laterally on the jaw but had minimal stress across the midline. This “ridge” area of low stress was not present to the same extent in LFs and could represent a structural adaptation for coping with load across the relatively narrow width of the mandible in TRC. Comparatively, the LFs also exhibited higher stress laterally and in the dentary region than for the first two scenarios but the stress patterns in this area were not as intense as in TRC.

Females had a higher level of stress across the mandible than the males. This pattern was particularly evident in the TRC female which showed high levels of stress (>7 MPa) across the mandible for all loading scenarios. Both sexes were similar in how they dissipate stress with both LF males and females having low levels of stress across the dentary region and the “wings” during loading scenarios using 75% and 100% of the jaw width, and both TRC males and females had a reinforced “ridge” along the midline.

F2 hybrids showed high stresses across the articular web on both sides of the mandible for all loading scenarios and the stress patterns for each scenario for both hybrids were similar to the TRC female. However, the hybrid female showed very little stress across the dentary region when loading was across the full width of the jaw but possessed a similar stress pattern to the TRC male for the loading scenarios where the force was applied at 50% and 25% of the width. The hybrid female showed very little stress across the midline whereas the hybrid male showed major asymmetry in stress patterns across the mandible despite asymmetry in mandible shape accounting for less than 14% of the variation in the shape.

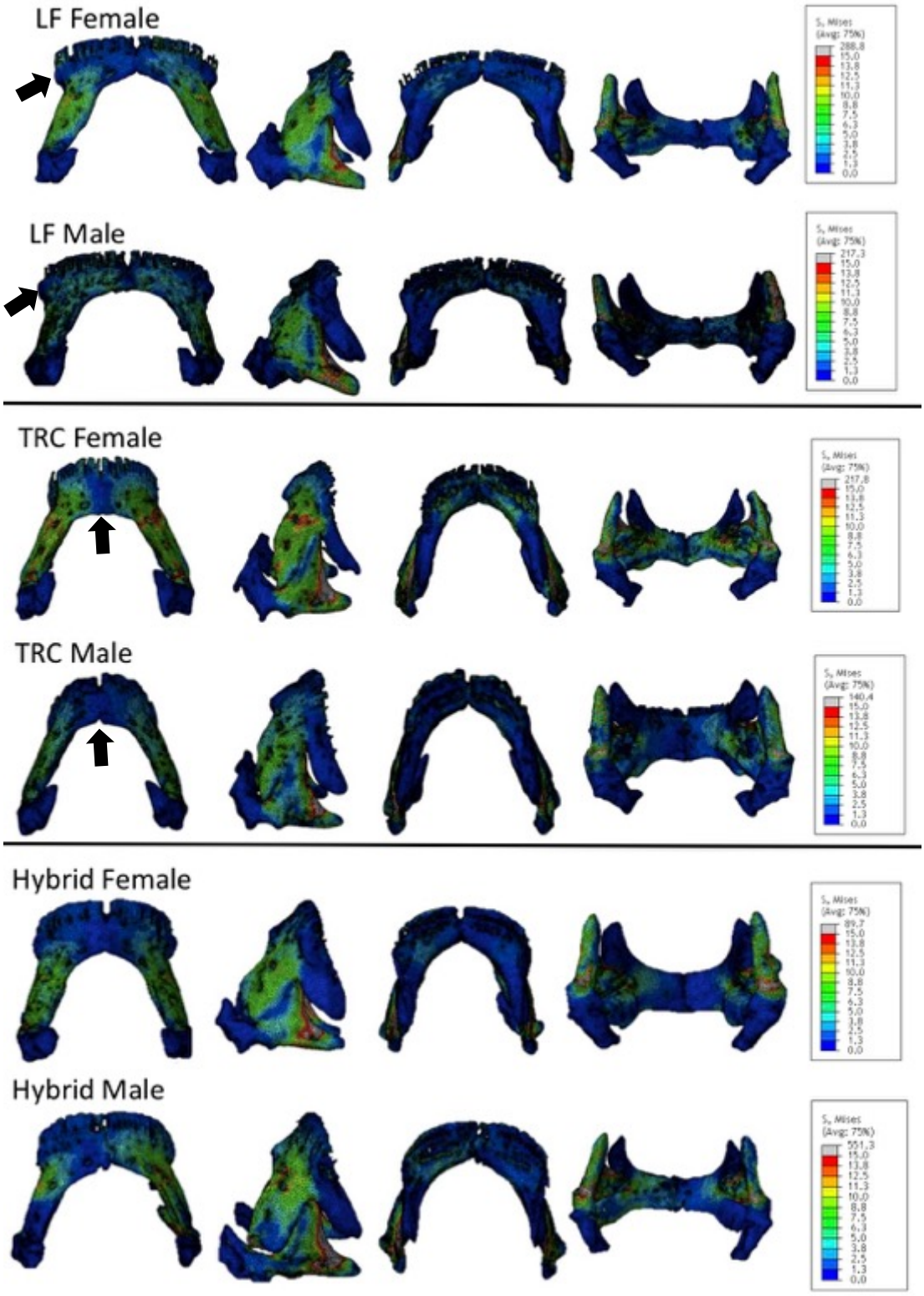

Figure 3—6: Results for each of the models for the 100% loading scenario shown from multiple angles (ventral, left lateral, dorsal and posterior views). The colours displayed on each model are the finite element analysis (FEA) results and represent a gradient of stress from low values (blue) to high (red); grey represents the maximum stress value (in MPa). In each Figure, the colour scale for the Von Mises stress is consistent across all models to allow for comparison; as grey is the maximum stress value this is variable across each model. Black arrows indicate the "ridge" for the *Tropheops* "Red Cheek" (TRC) and the "wings" in the *Labeotropheus fuelleborni* (LF).

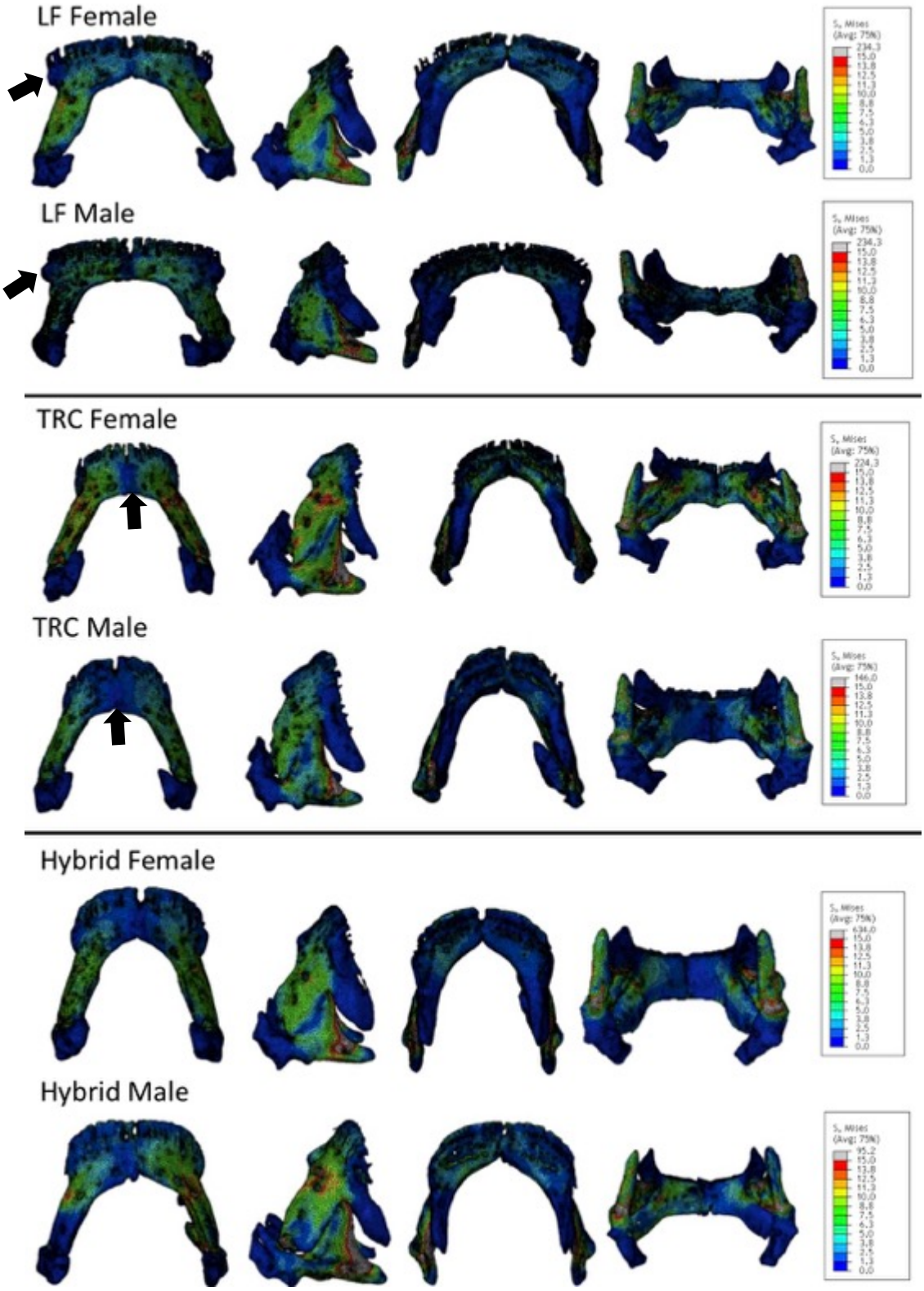

Figure 3—7: Results for each of the models for the 75% loading scenario shown from multiple angles (ventral, left lateral, dorsal and posterior views). The colours displayed on each model are the finite element analysis (FEA) results and represent a gradient of stress from low values (blue) to high (red); grey represents the maximum stress value (in MPa). In each Figure, the colour scale for the Von Mises stress is consistent across all models to allow for comparison; as grey is the maximum stress value this is variable across each model. Black arrows indicate the "ridge" for the *Tropheops* "Red Cheek" (TRC) and the "wings" in the *Labeotropheus fuelleborni* (LF).

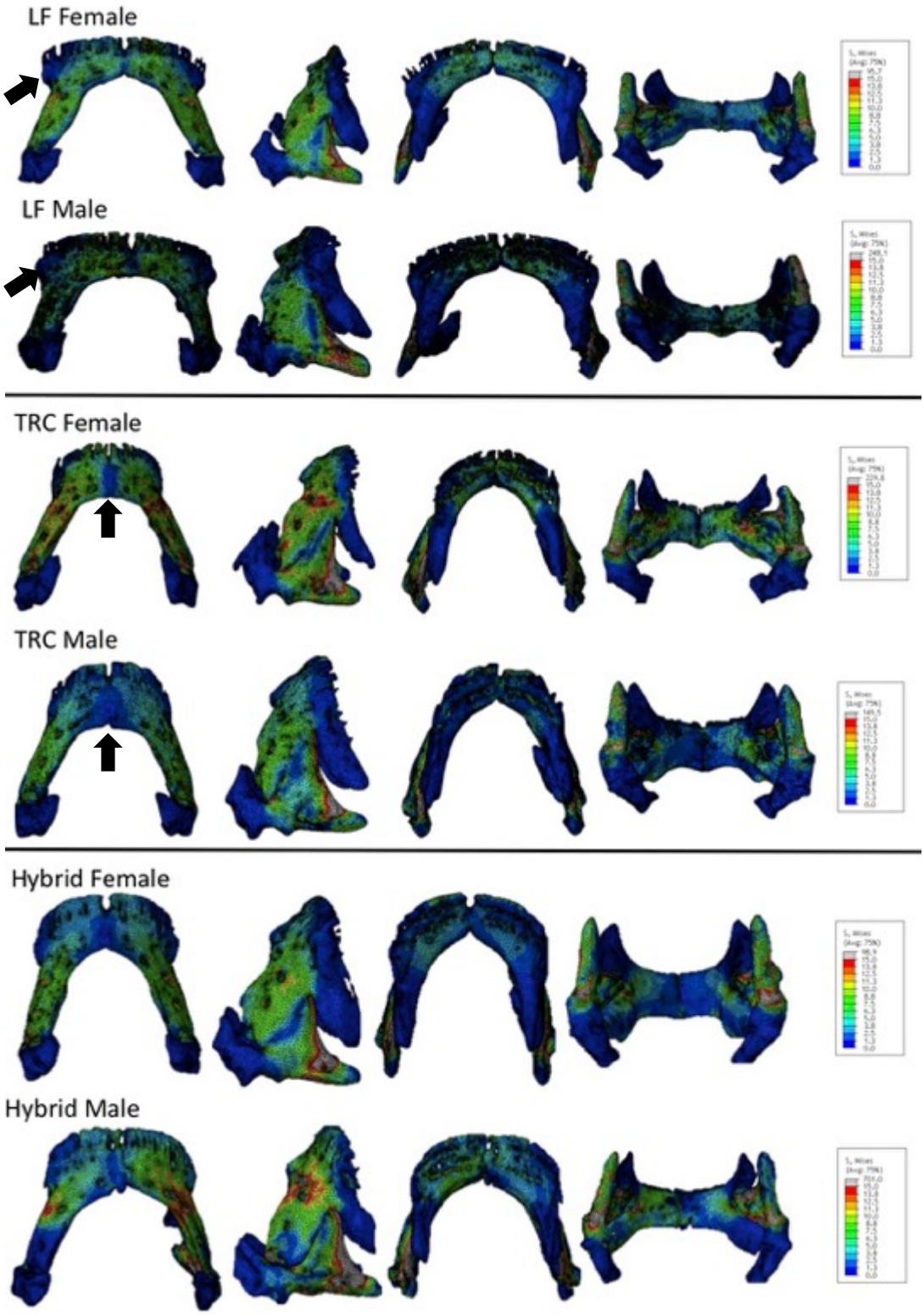

Figure 3—8: Results for each of the models for the 50% loading scenario shown from multiple angles (ventral, left lateral, dorsal and posterior views). The colours displayed on each model are the finite element analysis (FEA) results and represent a gradient of stress from low values (blue) to high (red); grey represents the maximum stress value (in MPa). In each Figure, the colour scale for the Von Mises stress is consistent across all models to allow for comparison; as grey is the maximum stress value this is variable across each model. Black arrows indicate the "ridge" for the *Tropheops* "Red Cheek" (TRC) and the "wings" in the *Labeotropheus fuelleborn*i (LF).

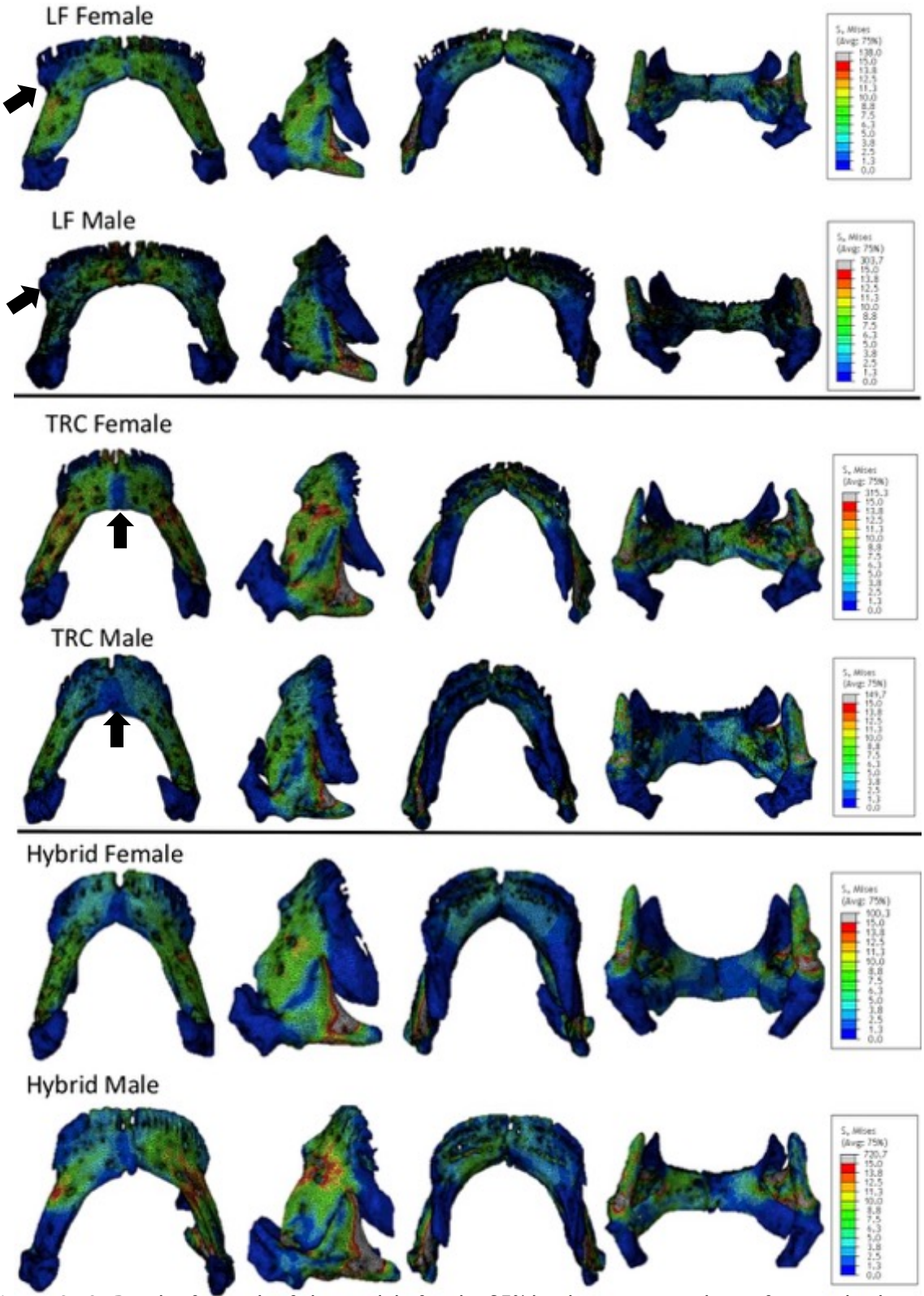

Figure 3—9: Results for each of the models for the 25% loading scenario shown from multiple angles (ventral, left lateral, dorsal and posterior views). The colours displayed on each model are the finite element analysis (FEA) results and represent a gradient of stress from low values (blue) to high (red); grey represents the maximum stress value (in MPa). In each Figure, the colour scale for the Von Mises stress is consistent across all models to allow for comparison; as grey is the maximum stress value this is variable across each model. Black arrows indicate the "ridge" for the *Tropheops* "Red Cheek" (TRC) and the "wings" in the *Labeotropheus fuelleborni* (LF).

3.5 Discussion

Although TRC and LF share a similar ecological niche, mandible shape and its ability to handle loading differs substantially. In the results presented here, the majority of mandible shape variation can be attributed to interspecific differences. Specifically, LF possess a much wider mandible than TRC which, when coupled with my FEA results, may confer a stronger ability to handle mechanical loading (**Figure 3-5**; Albertson and Pauers, 2018). Sexual dimorphism in mandible shape was evident in both species but with a much smaller effect than interspecific differences. Furthermore, sex-based variation in mandible shape reflected a similar trend to the interspecific differences with females possessing a much narrower mandible than the males. Given that a wider mandible increases the ability to bite and gather large amounts of algae, whereas a narrower mandible is better for suction feeding, such sexual dimorphism is likely to have ecological consequences (Albertson and Kocher, 2001).

There is evidence of sexual dimorphism in both parental species similar to that found in another species of Lake Malawi mbuna (*Maylandia zebra*) (McWhinnie and Parsons, 2019). It is likely that sexual dimorphism in mandible shape is widespread throughout the Malawi radiation. However, because I found that the level of sexual dimorphism for shape was the same for both species, this could represent an ancestral condition for cichlids, or that both species have reached an evolutionary maximum. This could also suggest that further sexual divergence in mandible shape may be limited. Despite this, if sexual dimorphism is an ancestral condition, then the presence of sexual dimorphism in cichlids may have provided a source of variation that facilitated an initial divergence into benthic and limnetic ecomorphs. Therefore, in Lake Malawi cichlids, if such sexual dimorphism is ancestral it may have biased early stages of adaptive radiation toward a benthic/limnetic habitat axis as has been suggested in threespine sticklebacks (McGee and Wainwright, 2013).

Divergence in feeding ecology between the species has likely been driven by how the mandible handles loading. Indeed, previous work has shown the ability of the neurocranium to resist biting forces on the vomerine process of the skull with

the steeper face of LF displaying lower stress than *Maylandia zebra* (MZ) which possesses a shallower face for suction feeding (Cooper *et al*. 2011). The responses in the LF mandible also show they were relatively better at dissipating stress when loading which mimicked their foraging mode was applied. Part of this may be due to LFs possessing a much wider jaw than TRC and the extended "wings" which protrude from the dentary region, and likely represent a unique foraging adaptation in *Labeotropheus*. This specialist adaptation to biting and scraping and the ability of the LF to handle stress efficiently could mean that this species 'shields' itself from environmental influence. Given that a benthic diet should create more external stress on the mandible, it could be expected that LF would experience a great deal of bone remodelling (Parsons *et al*. 2014). However, with such an efficient ability to dissipate external loading from feeding, remodelling might be negated in LF and could explain why they show slightly lower levels of morphological plasticity (Parsons *et al*. (2014), and investigated in **Chapter 4**).

Foraging tactics likely have a strong influence on patterns of mechanical loading. As TRC employs a nipping technique to pluck pieces of algae (Albertson, 2008), they would be unlikely to use the full width of the jaw like LF to contact food. In correspondence with this, the TRC FEA models showed minimal stress across the midline in all loading scenarios. This feature became particularly prominent when loading was applied that mimicked their foraging tactics. Given that this midline efficiency was not evident to the same extent in either of the LF models, this could be due to a structural adaptation. Indeed, TRC exhibited a 'ridge' of enhanced bone deposition across the midline which may be responsible for this increased efficiency. As previously mentioned, mechanical loading on bone induces remodelling through Wolff's Law (Wolff 1892; Chen *et al*. 2010). Therefore, in TRC, this area across the midline has perhaps been historically subjected to increased remodelling of bone as a result of the feeding method employed. Given that LF and TRC specimens were both wild caught or first-generation lab reared and had been fed the same food for long periods, this suggests that these potential adaptations are now part of the normal developmental process in both these species. When such an environmentally induced trait becomes part of normal development it suggests that genetic

assimilation has taken place (Pfennig *et al*. 2010). Similarly, in ostriches the callouses develop in their feet as part of normal development prior to hatching. Thus a trait which would normally be considered as one requiring environmental induction has now become a part of the genetic architecture and the phenotype (Gilbert, 1991).

Females performed comparatively worse at handling mechanical stress in both species. As the force to surface area ratio was the same for all models, the higher levels of stress indicate that the female mandible in both species would be unable to cope with the same force as the males. This could be due to the different uses for the mandible between sexes, with males showing high levels of aggression and fighting amongst each other for mates. This fighting occurs by locking jaws and biting, in turn providing greater mechanical loading in males relative to females (Ribbink *et al*. 1983). Mouthbrooding by females is also likely to place functional constraints on the craniofacial apparatus that males are not subject to. For example, a recent study reported that mouthbrooding in cichlids from Lake Victoria resulted in functional trade-offs for females (tkint *et al*. 2012). In this study, the necessity for space to mouth-brood in females has resulted in a better "biting" phenotype and a higher estimated bite force in males (tkint *et al*. 2012). Interestingly, that study also reported morphological differences between males and females similar to the sexual dimorphism in TRC and LF species. It is currently unknown how widespread sexual dimorphism is within the Lake Malawi radiation, but other studies have reported sexual dimorphism in the trophic morphology without considering the idea that this could be related to ESD (Oliveira and Almada, 1995; Herler *et al*. 2010). Such sexual dimorphism could increase the phenotypic variation in a population, and as my data shows, lead to functional divergence in response to loading on the mandible. Therefore, both reproductive and ecological differences could have additive effects that result sexual dimorphism.

The F2 hybrid models showed high stress patterns visible in the dentary region and the articular web of the mandible, with the female showing less stress than the male. In addition, the male showed asymmetry in the stress patterns in comparison to the female and this could be a result of the hybrid encompassing

phenotypic features of both the LF and TRC. Indeed, for all scenarios, it appears the F2 female handled stress better than the TRC female. Therefore, there could be wide variation in ability to handle loading on the mandible in the F2 hybrid population and this could explain why my hypothesis was not supported. The F2 female showed evidence of both a "ridge" and protrusions like the "wings" but this was not as noticeable in the male. In cichlids, hybridisation can result in novel transgressive morphologies which are remarkably different from either parental species (Albertson and Kocher, 2001; Genner and Turner, 2005). Furthermore, hybridisation can lead to a decrease in phenotypic integration in the craniofacial region which could therefore promote evolvability through the production of new phenotypes (Parsons, Son and Albertson, 2011). However, extreme variation in ability to handle stress could confer a negative functional advantage and explain why the Lake Malawi species complex has not collapsed. Given the wide variation in phenotypes, it could be that there is potentially wide variation in the ability to cope with stress and loading by the mandible in the hybrids; this requires further investigation beyond the scope of this chapter. Only two models were used for the FEA in this chapter, and it is likely further specimens would need to be modelled to fully explore the functional consequences of hybridisation in further detail.

The ability of cichlids to adapt to subtle differences in feeding ecology could explain the unprecedented success of the African Great Lakes adaptive radiations. Given that there are no major differences in bone density in mbuna cichlids (Cooper *et al*. 2011; Albertson, Cooper and Mann, 2012), this suggests the differences in ability to handle stress are adaptations reflected by the shape of the mandible and internal bone architecture (Albertson, Cooper and Mann 2012). Therefore, analysing variation within the internal bone architecture in the LF and TRC mandible would be an informative area for future research, and along with shape variation, could explain differences in ability to handle stress. This would also be a fruitful area for research in terms of sexual dimorphism as potential differences in internal bone architecture could provide further support for ESD.

3.6 Conclusions

Understanding the relationship between form, function and ecology is a key component of adaptive divergence research and exploring variation at an interspecific and intraspecific level can help increase our understanding of divergence. The results presented in this chapter highlight that both LF and TRC possess different structural and morphological adaptations for dissipating stress during feeding. As for the hybrids, they showed similar stress patterns to the TRC female, but with males showing an asymmetrical stress pattern with some areas of the mandible showing more stress than in the female; it is likely that there is a wide variation in ability to handle stress and this could confer a negative advantage within the radiation. As for sexual dimorphism, stress patterns also differed between males and females within each species indicating that males are able to cope with greater external forces than females. As males had a mandible better suited for biting and would likely experience more force during foraging, this lends weight to the idea of adaptive divergence between the sexes within the Lake Malawi cichlid radiation. The functional and morphological trends reported in this chapter suggest the differences between the sexes are ecologically relevant suggesting a role for ESD in the Lake Malawi radiation. However, it is important to note that the sexual dimorphism in the mandible may be have resulted from a combination of divergence in reproductive behaviour and foraging. Nonetheless, as adaptive radiation could be a combination of different levels of small-scale variation, sexual dimorphism could be a key contributor to this process, and this should be held in higher consideration for adaptive radiations.

Chapter 4: Testing for Sexual Dimorphism in Phenotypic Plasticity of Craniofacial Shape and Functionally Relevant Traits in African Cichlids

4.1 Abstract

Phenotypic plasticity is the ability of a genotype to produce multiple phenotypes in response to different environmental conditions and has been suggested as playing a key role in adaptive divergence (Wimberger, 1992; van Snick Gray and Stauffer, 2004; Pfennig *et al*. 2010). To assess phenotypic plasticity in an attempt to understand the contribution of plasticity to the possibility of ecological divergence between the sexes, a diet treatment experiment was conducted using two species (*Labeotropheus fuelleborni* and *Tropheops* "Red Cheek"). Plasticity in craniofacial shape and three functionally important traits were measured. These experiments did not show evidence of sex-based differences in plasticity for either species in any of the traits measured. This suggests mouth-brooding does not constrain plasticity in females as hypothesised and that ecological divergence between sexes does not rely on phenotypic plasticity. This could also mean that ecological sexual dimorphism is not important to the radiation, but the results presented in **Chapter 3** contradict this idea. The species did not markedly differ in plastic response in craniofacial shape which also differs from the hypothesis. Jaw protrusion showed a plastic response in both species but there were no differences between treatments in the other two traits measured suggesting that plasticity is focused in the area which directly interacts with the environment. Phenotypic plasticity has contributed to the variation present in the radiation and although sexes differ in craniofacial shape, there is no evidence of an interaction between the two.

4.2 Introduction

A fundamental goal of evolutionary biology is to understand how adaptive phenotypic variation arises. Recently, evolutionary thinking has expanded beyond the traditional view of genetic determinism as the driver of evolution to include environmental factors such as niche construction and phenotypic plasticity as important sources of adaptive variation (Laland *et al*. 2015). Plasticity is defined as the ability to produce multiple phenotypes from a single genotype in response to environmental conditions. Plasticity is commonly seen as provider of variation in the steps during the process of adaptive divergence and radiation (Pfennig *et al*. 2010). For example, the "flexible stem" hypothesis posits that adaptive divergence begins with plastic responses in ancestral populations that set the direction for further divergence (West-Eberhard 2003); this is supported with evidence from threespine sticklebacks (*Gasterosteus aculeatus*) (Wund *et al*. 2008) and African cichlids (Parsons *et al*. 2016).

While plasticity is usually considered as a contributor toward broad adaptive processes it is underappreciated how it might also contribute towards different levels of divergence. For example, sexual dimorphism is a common occurrence within vertebrates that could be enhanced by plasticity. Related to this ecological sexual dimorphism (ESD), whereby adaptive divergence evolves between sexes resulting in the occupation of different ecological niches (Shine, 1989), can evolve and form adaptive variation that is nested within broader patterns of ecological divergence (Foster, Scott and Cresko, 1998; Riopel, Robinson and Parsons, 2008; Parsons *et al*. 2015). However, an ecological cause for sexual dimorphism is challenging to show because it is difficult to separate from size variation between sexes, different nutritional requirements, sexual selection, or differences in reproductive output (Slatkin, 1984; Shine, 1989; Bolnick and Doebeli, 2003). Nonetheless, there are some clear examples of ESD recorded in snakes (Camilleri and Shine, 1990; Houston and Shine, 1993; Vincent, Herrel and Irschick, 2004), hummingbirds (Temeles, 2000; Temeles, Miller and Rifkin, 2010), and Caribbean *Anolis* lizards (Butler and Losos, 2002; Butler, Sawyer and Losos, 2007). Indeed, in the case of threespine sticklebacks, head shape can have little overlap between the sexes, with adaptive variation

associated with sexual dimorphism exceeding differences between ecological species in some populations (Aguirre *et al*. 2008; Aguirre and Akinpelu, 2010; Cooper, Gilman and Boughman, 2011). Therefore, given that phenotypic plasticity is viewed as a general contributor to adaptative divergence, it is reasonable to predict that plastic responses are also sexually dimorphic.

Changes in trophic morphology are key to many cases of ecological adaptation. Ecology can often be inferred from craniofacial morphology, but analysis of functionally relevant traits can more precisely assess adaptive responses (including plasticity and ESD) to the environment. For example, ecological adaptation along a benthic/limnetic habitat axis is characteristic of many different fishes (Wainwright, 1996; Adams and Huntingford, 2002; Rundle, 2002; Cooper *et al*. 2010). A steep craniofacial profile with short jaws is a more benthic phenotype as it confers an advantage for powerful bites, whereas a sloping profile with long jaws facilitates the fast movements required for suction feeding in a pelagic habitat (Cooper *et al*. 2010). Adaptation to these habitats is often assessed through direct functional assessments of feeding performance that are correlated with diet and prey use (Wainwright 1988). Alternatively, measurements from relevant anatomical traits can be used indirectly to infer functional performance based on biomechanical principles (Wainwright and Richard, 1995).

In fishes, jaw protrusion is highly relevant to feeding kinematics and can be used to predict suction feeding ability. Furthermore, it has been used extensively to explore the link between morphology and ecology in damselfish (Cooper *et al*. 2017), sticklebacks (McGee and Wainwright, 2013), and cichlids (Hulsey and García De León, 2005; Matthews and Albertson, 2017). Specifically, limnetic foragers have greater jaw protrusion than benthic foragers which aids in the capture of food from the water column by increasing suction abilities (Motta, 1984; McGee, Schluter and Wainwright, 2013; Matthews and Albertson, 2017). Feeding kinematics can also be influenced by plasticity as in the Lake Victoria cichlid *Neochromis greenwood* when raised on either an algal or zooplankton diet (Bouton, Witte and Van Alphen, 2002). In this case, fish raised on the algae treatment possessed a greater bite force, inferred through measures of the

musculature attached to the mandible, and through an increased angle between the ascending and dentigerous arms of the maxilla (Bouton, Witte and Van Alphen, 2002).

Other traits of importance to fish feeding adaptations and function are the retroarticular (RA) process of the mandible, and the interopercle (IOP) link, which extends from the IOP bone to the insertion of the IOP ligament on to the RA. The IOP directly transmits motion to the mandible through the interoperculomandibular ligament which inserts on to the posterior point of the RA of the mandible (**Figure 3-1**; Westneat, 1990). Therefore, both traits form two primary links in the teleost opercular four-bar linkage model with lengthening and shortening of these links being highly relevant for functional predictions (Hu and Albertson, 2014, 2017b). For example, a short RA and long IOP results in a reduction in the mechanical advantage of jaw opening (MA_O) which leads to a faster jaw rotation during opening; this is associated with suction feeding (Hu and Albertson, 2014; Westneat, 2003). Mechanical advantage relates to the force transmission capability of fish jaws and is often used as a means to identify differences between benthic and suction feeders (Barel, 1983; Westneat, 2003). A higher mechanical advantage results in greater force transmission which is more favourable for biting (Albertson *et al*. 2005). Conversely, a long RA and short IOP leads to a higher MA_O, but with a reduction in jaw opening speed, and usually occurs with a benthic mode of feeding (Barel, 1983; Westneat, 2003; Hu and Albertson, 2014).

It has been suggested phenotypic plasticity plays a key role in the rapid and explosive radiation of African cichlids (Wimberger, 1992; van Snick Gray and Stauffer, 2004). Given previous indications of phenotypic plasticity in African cichlids (Bouton, Witte and Van Alphen, 2002; van Snick Gray and Stauffer, 2004; Parsons *et al*. 2014, 2016), and evidence of sexual dimorphism in mandible and craniofacial shape (see data from **Chapter 2** and **3**, Parsons *et al*. (2015), and McWhinnie and Parsons (2019)), it is logical to hypothesise that plastic responses may differ between sexes. The potential for sexual dimorphism in plasticity links to constraints that females may face due to mouth-brooding. In line with this, tkint *et al*. (2012) calculated theoretical bite force in two Lake Victorian cichlid

species and found males had an increased bite force compared to females. Such dimorphism could be the result of different selective pressures on the sexes as females are mouth-brooders and males use their mouths for fighting (Ribbink *et al*. 1983; Konings 2001; Parsons *et al*. 2015). Therefore, constraints on females due to mouthbrooding could also act as a limiting factor for plastic responses and contribute to differences in ecology between sexes.

To assess the plasticity of craniofacial morphology in relation to sex and function, I performed a diet treatment experiment using the two focal species; *Tropheops* "Red Cheek" (TRC) and *Labeotropheus fuelleborni* (LF). I predicted that males would be more plastic than females as a consequence of the constraints placed on craniofacial morphology by mouthbrooding (tkint *et al*. 2012). Previous work has shown that a benthic diet results in a steeper face and shorter jaw compared to a limnetic diet which induces a more sloping face with longer jaws. Also, LF has previously been reported as possessing a more robust, less plastic, phenotype than TRC (Parsons *et al*. 2014). To extend these findings more directly toward ecological consequences I measured functionally relevant traits. Therefore, I also predicted that a limnetic diet would result in greater jaw protrusion, a shorter RA, and longer IOP link than the benthic treatment (Westneat, 1995; McGee, Schluter and Wainwright, 2013; Hu and Albertson, 2014; Matthews and Albertson, 2017), and that females would show less plasticity in these traits than males. By considering how multiple contributors to variation could influence adaptive radiation, this chapter examines phenotypic plasticity from a novel perspective.

4.3 Methods

4.3.1 Fish Husbandry and Rearing

Cichlid broods were collected in early 2017 for LF and late 2017 for TRC from females after 3 days post fertilisation (dpf). A total of 101 fish from ten broods were collected for TRC, and 115 fish from four broods were collected for LF. Each brood was raised separately in a 1L conical flask with 1-2 drops of

methylene blue to prevent fungus and an air stone to ensure the embryos were adequately aerated. Water changes were conducted regularly with broods being raised in the same water as their parents in the University of Glasgow Aquaria facility. At around 20 dpf the yolk was nearly completely absorbed, and broods were moved into a small tank (~25L) to feed independently. Each brood was raised in a separate tank and fine mesh was placed over the outflow pipe to prevent fish from escaping. After a further 4-6 weeks, fish were moved into a larger (~125L) tank. In total there were four treatment tanks for each species; two for the benthic treatment and two for the limnetic. To limit potential effects from density, each family was divided approximately equally across four treatment tanks with each tank containing between 22-26 fish. Each tank contained the same enrichment (no substrate on the bottom and the same number of ceramic pots and tubes) and used the same water supply.

4.3.2 Diet Treatment Experiment

To test the impact of different biomechanical demands on development, treatment groups were fed one of either a limnetic or benthic diet based on previous methods (Parsons *et al.* 2014, 2016). The content of food was kept the same to limit the possibility of nutritional effects on morphological plasticity (Wimberger 1993). A limnetic treatment, given to two of the groups, consisted of a ground mixture of flake food, algae wafer and freeze-dried daphnia which was then sprinkled into the water column to elicit suction feeding. The benthic treatment, given to the two treatment groups was the same mixture but air dried on lava rocks. During feeding these rocks were placed at the bottom of the tank to elicit a biting mode of feeding. Each treatment tank was fed twice daily for approximately 6-7 months until fish were within the size range of a mature cichlid (approximately 4-8 cm SL) and sexual dimorphism in colouration and spawning activity had begun. Fish smaller than this were not included in downstream analysis as they were difficult to dissect (n = 4). Due to space constraints, only one species could be on the plasticity experiment at a time. Once the treatments ended, fish were euthanised following UK Home Office

Schedule 1 guidelines, labelled, and stored in 10% neutral buffered formalin (NBF). Fish were sexed by assessing the internal anatomy, colouration and venting; male colouration is bolder, and their anal and genital vents are the same size whereas female colouration is dull, and the genital vent is larger in size than the anal vent (Moore and Roberts, 2017).

4.3.3 Morphometrics

Following fixation, the craniofacial region was dissected to reveal musculature and allow functionally relevant landmarks to be collected for geometric morphometrics. Craniofacial landmarks were selected based on previous work (Cooper *et al*. 2010; Parsons *et al*. 2016) to ensure that they were relevant to the evolution and functional anatomy of cichlids. Fish were secured to a wax dish with a scale and ID tag and photographed laterally from a fixed distance, with their mouth closed, using a mounted Canon EOS 1100D camera (Canon (UK), Surrey) and then returned to 10% NBF for storage.

For landmark digitisation, the tps suite of software was used (available at: http://life.bio.sunysb.edu/ee/rohlf/software.html). Prior to digitisation and to reduce intra-observer variability, the ID tags were removed from the images and the photographs were randomised so that landmarks could be added to the images blind to the treatment group. Landmarks were digitised for each specimen (**Figure 4-1**) with a scale factor measured for each image. Following digitisation, the landmarks were then analysed in R version 3.4.1 (R Core Team 2017) using the geomorph package (Adams and Otárola-Castillo, 2013; Adams, Collyer and Kaliontzopoulou, 2019). Before any analysis could take place, the landmarks were subjected to a Procrustes superimposition that translated, rotated and scaled the landmark configurations to a common centroid size, position and orientation (Zelditch, Swiderski and Sheets 2012a). Procrustes coordinates were then used for all downstream analyses. To explore allometric patterns in the two species, *procD.lm* was used to assess the relationship between size and shape, using a linear model, in the two species. Then, *plotAllometry* was used on the model fit to visualise and compare the allometric

patterns in the two species. As the allometric slopes were not parallel, a common allometric regression could not be applied and allometric effects remained in the shape coordinates (Klingenberg, 2016).

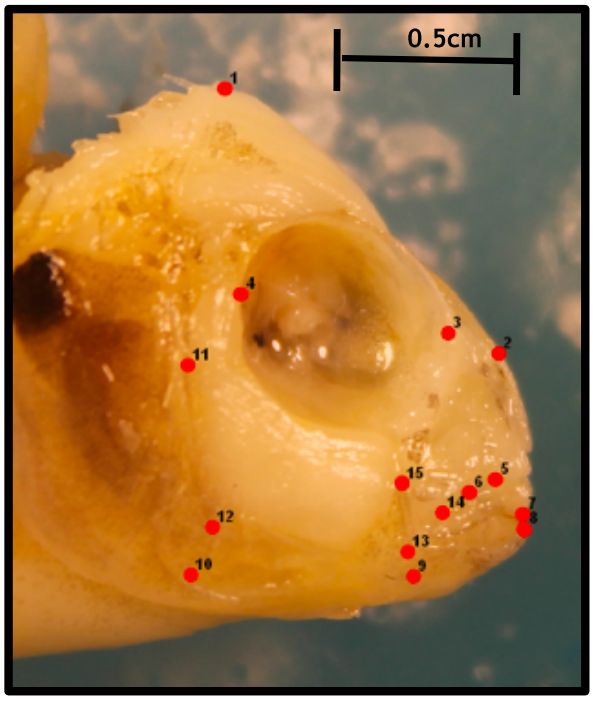

Figure 4—1: The landmarks selected for morphometrics based on functional and ecological relevance (Cooper et al. 2010, Parsons, Son and Albertson, 2011, and Parsons *et al*. 2016). Landmarks represent the following anatomical locations: 1) Dorsal end of the occipital crest; 2) Posterior tip of the premaxilla; 3) Anterio-ventral point of eye socket; 4) Posterio-ventral point of eye socket; 5) Maxillary-palatine joint; 6) Muscle insertion on the maxilla ; 7) Tip of the tooth on the pre-maxilla; 8) Tip of the tooth on the mandible; 9) Retroarticular of the mandible; 10) Posterio-ventral corner of preopercular bone; 12) Origin point of muscle insertion on the pre-opercular; 12) Posterio-ventral corner of muscle origin; 13) Articular-quadrate joint; 14) Maxillary-articulation joint; 15) Muscle insertion on the articular process of the mandible.

To simultaneously assess the effect of species, diet, sex, and their interactions on shape a Procrustes ANOVA, using the *procD.lm* function, was conducted on Procrustes coordinates. To explore shape changes in relation to plasticity and test whether specimens could be classified using *a priori* groupings (treatment and both sexes of each treatment for each species), a discriminant function analysis (DFA) was conducted on all of the PC scores, generated by *plotTangentSpace* from the Procrustes shape coordinates, using the *lda* function

in the MASS package (Venables and Ripley 2002). To visualise shape changes relating to diet between sexes and species, the function *shape.predictor (*also from geomorph) was used. This function estimates shape configurations, using the Procrustes shape coordinates, to produce deformation grids showing shape change and is based on a linear factor, in this case the linear discriminant axis from the DFA.

A useful measure to test for differences in variance among samples or for testing differences in mean shape is utilising the partial Procrustes distance (PPD). As described by Webster and Sheets (2010), the partial Procrustes distance (PPD) is a morphometric distance and is the amount of difference in shape between two landmark configurations; variation within a group can be measured as the average PPD from the mean configuration. Therefore, to test the hypothesis that species and sexes would differ in plastic response to diet treatments, PPD were calculated and compared using TwoGroup from the IMP suite of software (available at: http://www.philadb.com/an-behav/imp/) with 900 bootstraps. The software calculates the PPD between two groups of landmark configurations and provides a 95% confidence interval for this distance. Four groups can be compared at same time. Firstly, the PPD between groups 1 and 2, and groups 3 and 4 is calculated. Then to compare the difference between the two PPDs relative to one another, a bootstrapping procedure is used to test for significance and then a confidence interval for the difference in distances between the pairs is provided; if zero is not part of the distribution then the null hypothesis that the distances are not different can be rejected (Webster and Sheets, 2010). In the context of this chapter, three different tests were conducted. Firstly, the PPD between the TRC benthic and limnetic groups was calculated and compared with the PPD between the LF benthic and limnetic groups. Then the PPD between the TRC benthic and limnetic males was calculated and compared with the PPD between the LF benthic and limnetic males. Finally, the PPD between the TRC benthic and limnetic females was calculated and compared with the PPD between the LF benthic and limnetic females.

4.3.4 Measurement of Functionally Relevant Traits

To assess plasticity on functional traits fish were photographed from a lateral view with their mouth open following McGee, Schluter and Wainwright (2013) and Matthews and Albertson (2017). To allow the jaws to be opened easily, fish were first digested in a 1% trypsin solution (1g of trypsin in 350ml saturated sodium borate and 650ml distilled water) following Pothoff (1984) and left to rock gently. The trypsin digestion stage allowed for a more natural range of movement from muscles and ligaments than what results from formalin storage (McGee, Schluter and Wainwright, 2013). The jaw was opened by first securing the fish on a wax dish and using forceps to gently relax the ligaments and open the jaws, and then by using a metal rod to press onto the neurocranium to encourage the jaws to open as naturally as possible (McGee, Schluter and Wainwright, 2013). Lateral photographs with the mouth open and upper jaw protruded were captured using a Canon EOS 1100D camera. Two landmarks were digitised on each photo (**Figure 4-2A**), representing the length of jaw protrusion; this was the linear distance from the proximal (landmark 1) to the distal (landmark 2) point of the premaxilla (Matthews and Albertson 2017). The distance between the two landmarks was calculated and then standardised for size using a linear regression of the standard length for each specimen. The size-standardised residuals were then used in an ANOVA using species, treatment, sex and their interaction as explanatory variables.

To test the prediction that the IOP and RA would be more plastic in response to diet treatment in males and in TRC, fish were taken through a clearing and staining protocol following Pothoff (1984). Firstly, fish were stained with alizarin red at a ratio of 1:40 in 1% potassium hydroxide solution (KOH) to highlight areas of bone to make it easier to identify the IOP link and the RA. After staining, fish were stepped through a series of KOH and glycerol changes following Pothoff (1984) to clear excess stain and then photographed again with the mouth closed. As before, landmarks were placed to identify these areas (**Figure 4-2B**) and inter-landmark distances were calculated for each trait as described above. However, the fixed link of the opercular four-bar was also measured (shown in black in **Figure 4-2B**) and used to standardise for size. Both the RA and IOP were

calculated as a ratio of the fixed link following Hulsey and García De León (2005) as this is a relevant way of removing size variation from the measurements of links in the four-bar (Hulsey and Wainwright, 2002). The ratios were then used in separate ANOVAs using species, treatment, sex and their interactions as factors.

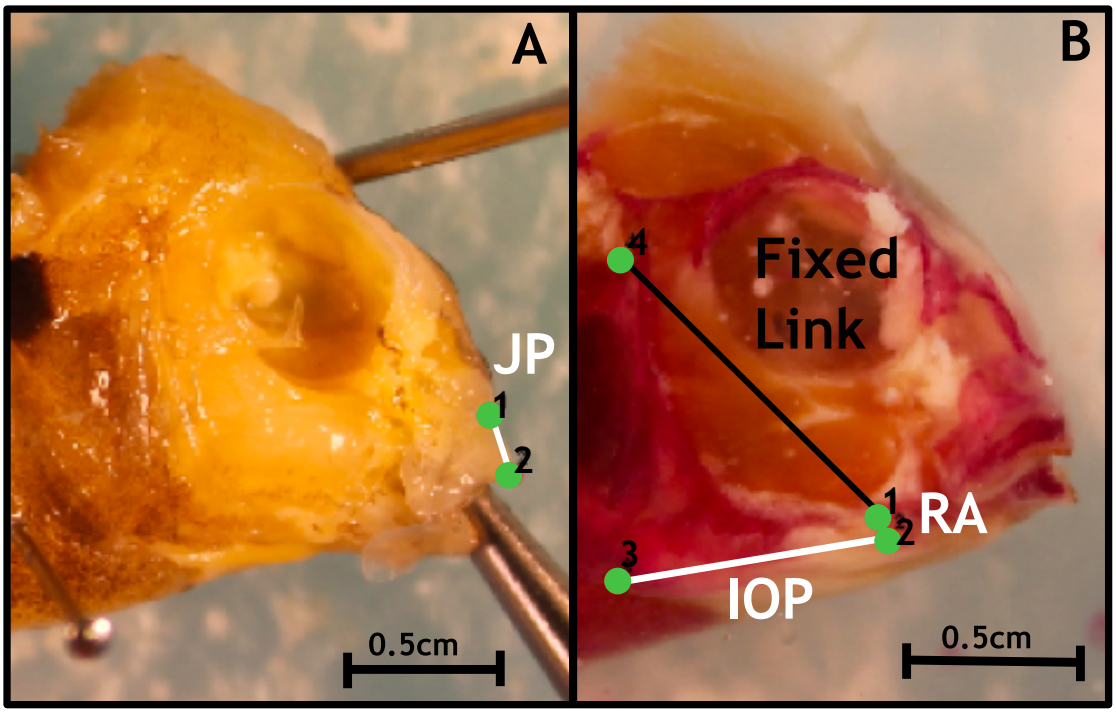

Figure 4—2: The three functionally relevant traits measured (**JP** = jaw protrusion; **IOP** = interopercular link; **RA** = retroarticular process) as shown on TRC specimens, are indicated by a white line, and the fixed link (used as a ratio to factor out size for the IOP and RA) of the opercular four-bar linkage is indicated in black. A scale bar is added for reference for each image. **A** is a trypsin digested specimen of TRC, and **B** is a TRC specimen after Alizarin staining and KOH clearing. The landmarks used in A are: 1) proximal point of the premaxilla and 2) distal point of the premaxilla. The landmarks used in B are: 1) articular-quadrate joint; 2) retroarticular of the mandible; 3) posterior edge of the IOP bone; 4) opercle-neurocranium joint.

4.4 Results

4.4.1 Morphometrics of Phenotypic Plasticity

Both species showed evidence of phenotypic plasticity in craniofacial shape as a result of the two treatments; the final samples sizes were 94 fish for TRC and 101 fish for LF. However, a significant interaction showed that plasticity differed between species (**Table 4-1**). For the discriminant function models 91% of the

benthic and 92% of limnetic TRC specimens were classified correctly whereas classification rates were slightly lower for LF where 88% of benthic and 84% of limnetic specimens were correctly classified (**Figure 4-3**). The partial Procrustes distance (PPD) between each treatment was similar in each species with no significant difference when the two species were compared (CI of difference between species treatment group pairs = -0.013 to 0.012). Similar shape changes occurred for each species with the benthic treatment resulting in a steeper face relative to the limnetic treatment and the mandible appeared slightly shorter in the benthic specimens in comparison to limnetic treatments (**Figure 4-3**).

Table 4-1: Summary of output from the Procrustes ANOVA model for assessing phenotypic plasticity and sex effects on craniofacial shape. P values were obtained through permutation procedures.

Factors	DF	Sum Sq	Mean Sq	R Sq	F	Z	P
Species	2	0.21723	0.217226	0.21764	58.1864	7.8778	0.001**
Treatment	2	0.03991	0.039908	0.03998	10.6898	5.3844	0.001**
Sex	2	0.02546	0.025457	0.02551	6.8189	4.6977	0.001**
Species:Treatment	2	0.00772	0.007722	0.00774	2.0683	1.9495	0.030*
Species:Sex	2	0.00396	0.003963	0.00397	1.0615	0.4031	0.333
Treatment:Sex	2	0.00250	0.002498	0.00250	0.6690	-0.6718	0.752
Species:Treatment:Sex	2	0.00319	0.003194	0.00320	0.8555	-0.0448	0.530
Residuals	180	0.69812	0.003733	0.69946			

***P < 0.05; **P < 0.01, ***P < 0.001**

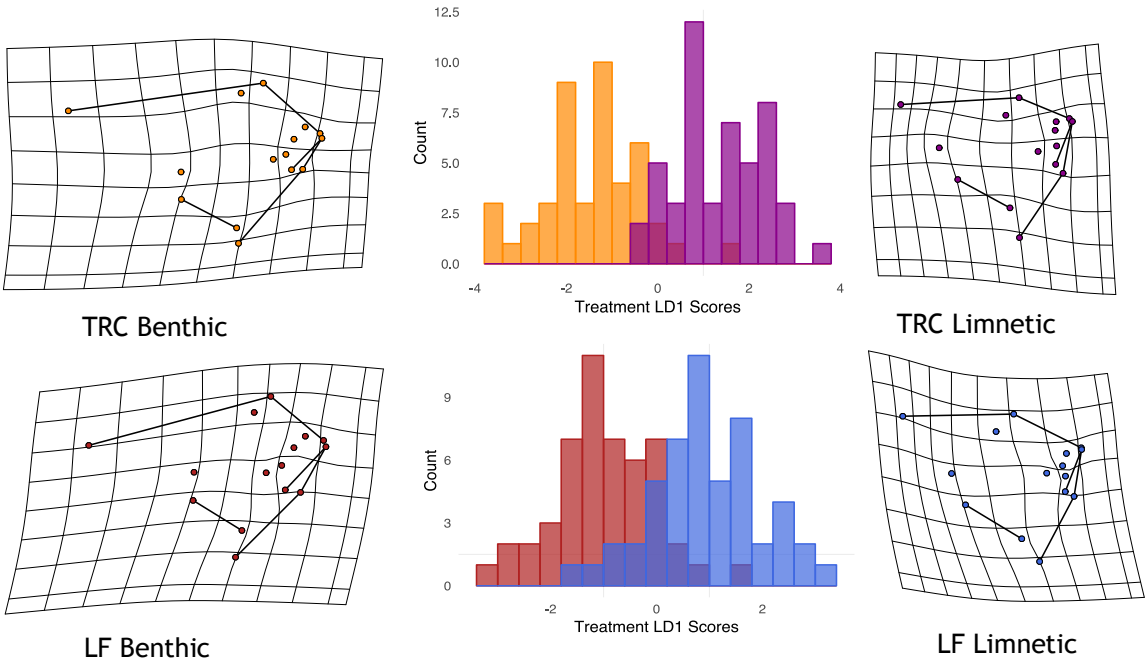

Figure 4—3: Frequency histograms displaying the classification rate of diet treatment with accompanying deformation grids depicting associated shape variation for each species. For *Labeotropheus fuelleborni* (LF) the benthic specimens are represented in red and the limnetic are in blue, while for *Tropheops* "Red Cheek" (TRC), the benthic specimens are represented in orange and limnetic in purple.

Table 4-2: The partial Procrustes distance and associated 95% confidence intervals between groups after 900 bootstraps between each treatment for both species.

Species	Groups	Partial Procrustes Distance	95% CI
LF	Benthic and Limnetic	0.031	**0.025 to 0.043**
	Benthic Males and Limnetic Males	0.033	**0.0250 to 0.048**
	Benthic Females and Limnetic Females	0.031	**0.025 to 0.047**
TRC	Benthic and Limnetic	0.032	**0.026 to 0.042**
	Benthic Males and Limnetic Males	0.034	**0.026 to 0.053**
	Benthic Females and Limnetic Females	0.033	**0.028 to 0.046**

Although there was sexual dimorphism in craniofacial shape this did not differ between species and there was no difference in plastic response between sexes for either species (**Table 4-1**). Classification success for sex based on DFAs was

similar for each sex and species (LF = 92% of benthic males, 93% of limnetic males and 96% of benthic females and 95% of limnetic females; TRC = 96% of benthic females, 92% of limnetic females, 100% of benthic males and 100% of limnetic males). In addition, there was no significant difference in PPD between the treatment groups for either sex within each species (**Table 4-2**; Difference between LF sex pairs= -0.019 to 0.014; difference between TRC sex pairs= -0.018 to 0.015). Shape changes were similar for each sex undergoing the benthic and limnetic treatments with benthic fish having a steeper profile than the relatively sloping profile of the limnetic fish (**Figure 4-4**). Together this shows that plastic responses did not differ between the sexes for either species.

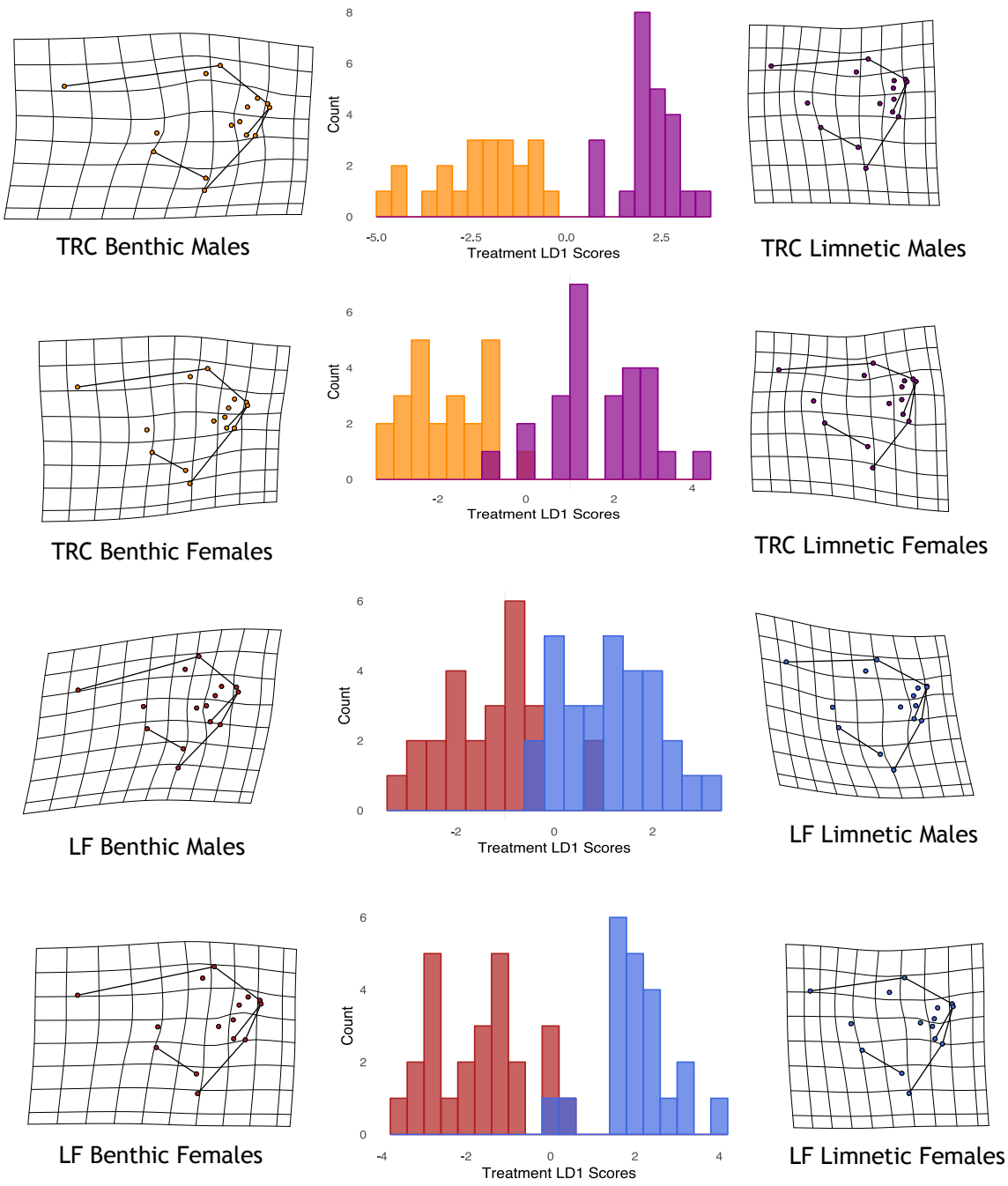

Figure 4—4: Frequency histograms for sex derived from DFA models using treatment as a grouping variable for each sex and species. For *Labeotropheus fuelleborni* (LF) the benthic specimens are represented in red with the limnetic in blue. For *Tropheops* "Red Cheek" (TRC), the benthic specimens are represented in orange with the limnetic in purple. The shape changes associated with each DFA model are depicted with deformation grids to the right and left of the histograms.

4.4.2 Plasticity of Functional Traits

For functional traits, the two species differed in jaw protrusion length (**Table 4-3**; **Figure 4-5**). Further, the ANOVA model revealed treatment effects, but no interaction between species and treatment (**Table 4-3**). For both species, jaw protrusion was greater for the limnetic treatment than for the benthic, but this difference did not appear as pronounced for LF where there was considerable overlap between the two treatments (**Figure 4-5**). There was no significant difference in jaw protrusion between the sexes or between any interactions. For the two other functional traits, the ANOVAs revealed no plasticity in length for the RA, however there was a significant difference between species in RA length and IOP length (**Table 4-3**). In addition, there was no difference in RA or IOP length between the sexes and no significant interactions involving sex its interactions (**Table 4-3**).

Table 4-3: The results of ANOVA models examining size-corrected jaw protrusion residuals (n = 178), relative retroarticular (RA) length (cm) (n = 192) and relative interopercular (IOP) length (cm) (n = 192). Asterisks denote statistically significant P values.

Trait	Factors	DF	Sum Sq	Mean Sq	F	P
Jaw Protrusion	Species	1	0.10193	0.10193	119.947	2e-16**
	Treatment	1	0.01756	0.01756	20.663	1e-05**
	Sex	1	0.00010	0.00010	0.120	0.729
	Species:Treatment	1	0.00183	0.00183	2.156	0.144
	Treatment:Sex	1	0.00006	0.00006	0.068	0.795
	Species:Treatment:Sex	1	0.00154	0.00154	1.813	0.180
	Residuals	171	0.14531	0.00085		
Relative RA	Species	1	0.03286	0.03286	188.363	2e-16***
	Treatment	1	0.00029	0.00029	1.643	0.202
	Sex	1	0.00010	0.00010	0.566	0.453
	Species:Treatment	1	0.00012	0.00012	0.681	0.410
	Treatment:Sex	1	0.00000	0.00000	0.010	0.921
	Species:Treatment:Sex	1	0.00019	0.00019	1.081	0.300
	Residuals	185	0.03227	0.00017		
Relative IOP	Species	1	0.02052	0.020515	20.669	9e-06**
	Treatment	1	0.00007	0.000067	0.067	0.7960
	Sex	1	0.00276	0.002759	2.779	0.0972
	Species:Treatment	1	0.00030	0.000298	0.301	0.5842
	Treatment:Sex	1	0.00000	0.000000	0.000	0.9912
	Species:Treatment:Sex	1	0.00016	0.000161	0.163	0.6872
	Residuals	185	0.18363	0.000993		

*P < 0.05; **P < 0.01, ***P < 0.001

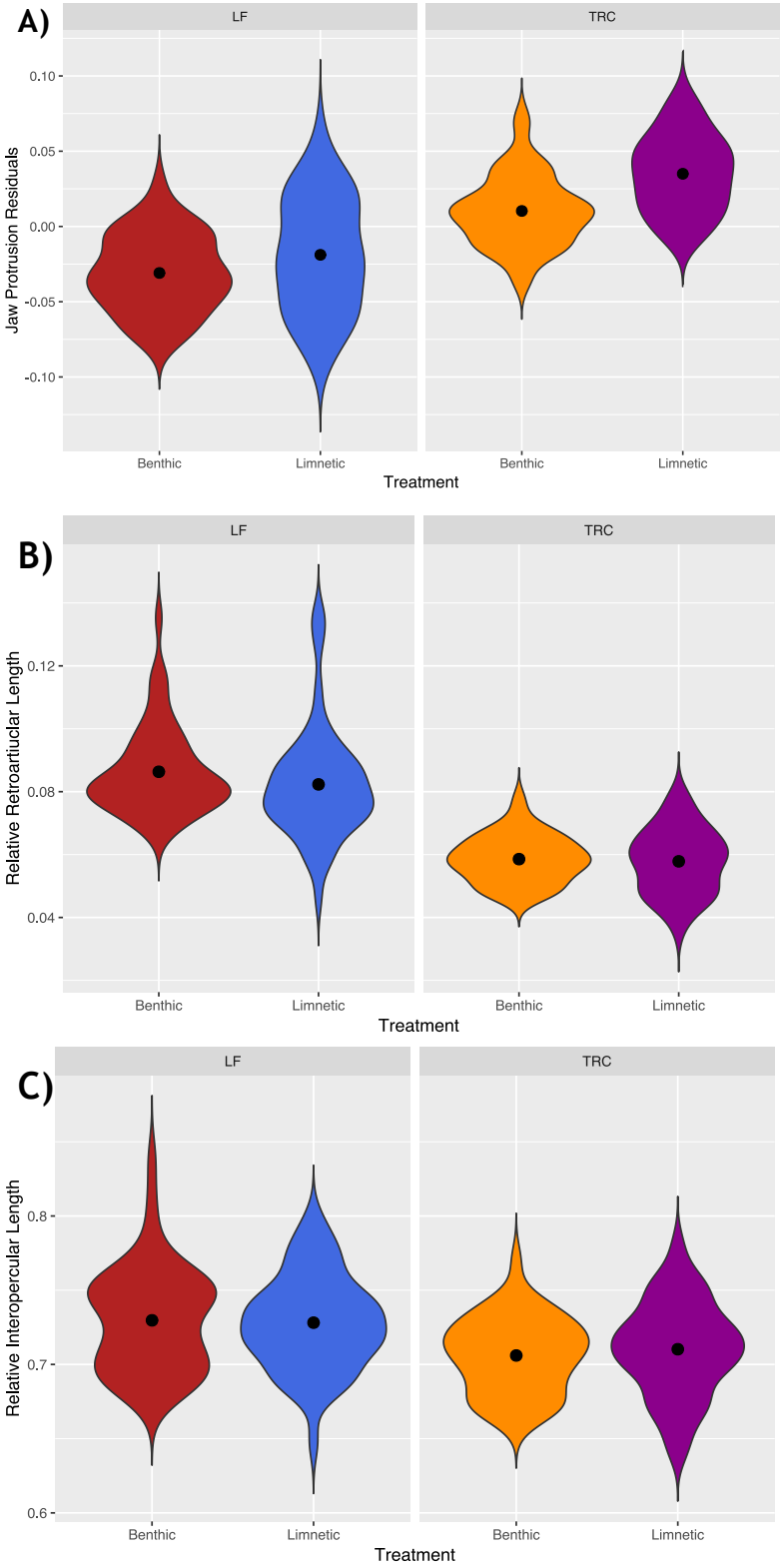

Figure 4—5: Comparison of functional morphological traits between cichlid species (*Tropheops* "Red Cheek" (TRC), and *Labeotropheus fuelleborni* (LF)) and benthic and limnetic foraging treatments after size correction. The distribution of the data for each trait is shown and the black dot represents the mean value for each trait. In panel **A)** jaw protrusion residuals are provided for each treatment and species (n = 177), while **B)** shows relative retroarticular (RA) length (cm) for each treatment for both species (n = 193) and **C)** shows relative interopercular (IOP) length (cm) for each treatment for both species (n = 193).

4.5 Discussion

I have approached phenotypic plasticity from the novel angle that it may differ with sex. In both cichlid species, sex differences in craniofacial shape were significant but not related to plasticity. This contradicts my hypothesis that males would be more plastic than females and suggests that sexual dimorphism in plasticity is absent despite evidence of ecologically relevant divergence between sexes in this group. This suggests that phenotypic plasticity is likely not important for the maintenance of ESD in this group and suggests that ESD would not be initiated, or at least extended by a plastic contribution as models for conventional adaptive divergence would suggest (Pfennig *et al*. 2010; Levis and Pfennig, 2016). It is possible that ecological divergence between the sexes in African cichlids is likely to be based on a wholly genetically determined process that is robust to environmental influences.

Both species exhibited phenotypic plasticity in craniofacial shape. However, while the difference in plasticity between the species was significant it was relatively small, with the interaction term accounting for only 0.77% of the variation. This differs from a previous investigation of plasticity in these species (Parsons *et al*. 2014) where TRC was substantially more plastic, but this could be due to my larger sample size or the use of a different LF strain. Nonetheless, a reduction in plasticity in LF could be due to their highly specialised phenotype for scraping algae and the general idea that increases in specialisation lead to reductions in phenotypic plasticity (Skulason and Smith, 1995; Parsons *et al*. 2014). Furthermore, LF exhibit accelerated bone deposition partly through increased *Wnt* signalling which establishes the craniofacial phenotype early on in ontogeny; this results in a more developmentally canalised phenotype which is likely more robust to environmental fluctuation (Parsons *et al*. 2014). However, other research has shown evidence of plasticity in LF in response to diet treatment (van Snick Gray and Stauffer, 2004). Therefore, it could be that there is population level variation in plastic responses especially as LF are one of the few widespread species along the rocky shoreline of Lake Malawi (Ribbink *et al*. 1983; Parsons *et al*. 2014).

Jaw protrusion, an important kinematic trait, showed a plastic response to diet in both species with benthic fish having a shorter jaw protrusion than limnetic-reared fish which would likely incur a reduced ability to suction feed (Motta, 1984; Waltzek and Wainwright, 2003). Overall, TRCs possessed greater jaw protrusion than LFs which would be expected given that LF is a specialised biter with an exaggerated fleshy snout which extends over the maxilla (Conith *et al*. 2018). Previously, it has been suggested that this flap over the maxilla results in a reduction in the ability to protrude the upper jaw to facilitate the transmission and generation of greater forces during feeding (Concannon and Albertson, 2015). Jaw protrusion was the only functional trait to show evidence of plasticity, making it especially interesting for future study. Given that it is arguably more directly linked to foraging, it may be adaptive to maintain greater levels of plasticity in this trait due to longer-term fluctuations in prey in the environment.

However, there was a lack of plasticity or dimorphism between sexes in jaw protrusion. Given that jaw protrusion is arguably one of the most important traits involved with adaptive divergence in fishes, and has been proposed as a key innovation in the evolution of vertebrate suction feeding, this could have substantial evolutionary consequences (Wainwright *et al*. 2015). In anadromous threespine sticklebacks, McGee and Wainwright (2013) reported sexual dimorphism in traits relevant to feeding mechanics, particularly jaw protrusion. In this case, it was suggested that ecologically relevant sexual dimorphism could enable sticklebacks to quickly adapt into limnetic and benthic eco-morphs when colonising new habitats. Given my findings, it may be that sexual dimorphism in cichlids does not represent the same underlying mechanisms found in wider patterns of adaptive divergence. Instead, ESD may provide an alternate form of divergence from a mechanistic perspective, that phenotypically resembles wider patterns of divergence between species. This idea is supported by cichlid males tending to possess a more “biting” phenotype relative to females with their “suctioning” facial shape, a pattern that is aligned with the main trajectory of divergence within lake Malawi (Parsons *et al*. 2015). To address these ideas, future research investigating the mechanisms of divergence at both a population-level scale, coupled with sexual dimorphism would be especially

enlightening. At this point the data suggests that ESD is not influenced by plasticity but it remains to be seen in other systems.

No evidence for phenotypic plasticity or sexual dimorphism was detected in either the RA or IOP functional traits. Given that sexual dimorphism in shape was apparent, this suggests that such variation can exist with minimal effect on some aspects of function. However, there may be some limitations in the power of my study to detect changes in these traits. As an algal scraper, LF would require a higher mechanical advantage, and therefore a relatively longer RA than TRC and my data matched this prediction. Similarly, a relatively shorter IOP is concomitant with biting (Hu and Albertson, 2014), but I found that LF had a significantly longer IOP than TRC, albeit with considerable overlap in length visible between the two species. The IOP has been measured and compared in these cichlids but instead of the length of the link, it was a ratio between the width and length of the bone (Hu and Albertson, 2014). Therefore, although an important component of the teleost opercular four-bar (Hu and Albertson, 2014), this suggests that measuring the length of this link is unreliable when the species have similar ecologies and therefore likely similar functional demands. Both the IOP and RA links in cichlids are influenced by *ptch1*, a member of the hedgehog signalling pathway (Roberts *et al*. 2011; Hu and Albertson, 2014). This pathway is key for cichlid craniofacial development, with increased expression in LF leading to a longer RA and shorter IOP relative to a suction-feeding generalist *Maylandia zebra* (Hu and Albertson, 2014). However, in more similar trophic morphologies like those possessed by TRC and LF, whereby both have short jaws relative to other members of the adaptive radiation, mechanisms may differ. *Tropheops* are still segregating alleles at this gene which has led to variation in RA length, and thus mechanical advantage of jaw opening (MA_O) (Roberts *et al*. 2011; Hu and Albertson, 2014). The more recently derived allele is associated with a more limnetic phenotype through a reduction in the RA length and MA_O (Roberts *et al*. 2011; Hu and Albertson, 2014). The derived allele found in TRC is less sensitive to foraging environment for the MA_O than the ancestral LF allele, suggesting that this trait is becoming less plastic which is consistent with my results (Parsons *et al*. 2016).

Finally, the lack of sexual dimorphism in plasticity in my results could also be due to its underlying mechanisms. In a study of the genetic basis of plasticity in a hybrid cross of LF and TRC, the authors only reported one trait out of nine that mapped to the sex determining region (Parsons *et al*. 2016). This was the ventral aspect of the fish extending from the mandible to the pelvic fins. Given that this included variation in the buccal cavity (where developing eggs are held), it could be suggested that plastic responses in this region would differ between sexes. Therefore, this aspect should be a consideration for future inquiry.

Whilst my data does not point towards sex-based differences in plasticity, there was clear evidence for sexual dimorphism in shape. It was expected that mouthbrooding would act as a functional and ecological constraint for females and cause sexual dimorphism in plasticity. This is because mouthbrooding likely involves a different set of functional requirements that may be at odds with foraging. Given that "biting" requires more force and mechanical advantage, the associated larger jaw muscles could reduce space required for mouthbrooding (tkint *et al*. 2012). Evidence that mouthbrooding has resulted in functional consequences is also suggested by superior bite force in males (tkint *et al*. 2012). Such consequences could drive different adaptive strategies between sexes in African cichlids and set limits on the range of phenotypes possible within their adaptive radiations.

As well as a lack of differences in plasticity between sexes, my results did not extend to functional differences between sexes therefore suggesting that ESD is not an important factor in cichlid sexual dimorphism and evolution. However, **Chapter 3** contradicts this interpretation with clear, sex-based differences in the ability of the mandible to handle external forces. Therefore, this could instead suggest that my current measures of function are inadequate for investigating ESD. This could be due to the relatively small changes that ESD may incur in comparison to species differences that these measures have been used to investigate in cichlids (e.g. Roberts *et al*. 2011; Hu and Albertson, 2014). Indeed, there is no clear relationship between the magnitude of change in a trait and the degree to which it affects fitness; work in African finches *(Pyrenestes*) has reported that a width change of 1mm in the mandible can

result in a 50% change in fitness (Smith, 1993; Parsons *et al*. 2011). Furthermore, in sticklebacks, where functional work has shown evidence of sexual dimorphism, in some populations ecological divergence between the sexes exceeds that between species pairs (Cooper, Gilman and Boughman, 2011; McGee and Wainwright, 2013). This is not the case in cichlids therefore this could mean that ESD is not an important component of the radiation. Nonetheless, this result does not dismiss the possibility of ESD in Lake Malawi cichlids given the results of **Chapter 3**.

4.6 Conclusions

For Lake Malawi cichlids, it has been suggested that plasticity is still actively evolving (Parsons *et al*. 2016). However, my evidence suggests that plastic responses are not sexually dimorphic and therefore not important for the maintenance of ecological divergence between the sexes. Given the differing selection pressures sexes likely face, and the trend for females to possess more of a "suctioning" phenotype suited to carrying eggs, it would be of interest to assess whether the absence of a sexually dimorphic plastic response has negative consequences for females and mouthbrooding (tkint *et al*. 2012; Parsons *et al*. 2015). Whilst the results suggest that plasticity is not an important factor in maintaining divergence between sexes in cichlids, this would be interesting to investigate in a system where there is strong evidence of ecological sexual dimorphism. In some populations of threespine sticklebacks where the sexual dimorphism is more important variation than between species pairs (Cooper, Gilman and Boughman, 2011), sexual dimorphism in plastic responses would be an enlightening topic to explore.

Chapter 5: General Discussion

5.1 Summary of Thesis

I have investigated various aspects of adaptive divergence in African cichlids, an exemplar evolutionary system, including genotype-to-phenotype relationships in the mandible, the ability of the mandible to cope with stress and loading, and phenotypic plasticity in craniofacial shape and functionally relevant traits. Whilst this thesis considers divergence between species, which is a common component of adaptive divergence research, I also investigated the possibility of ecological sexual dimorphism (ESD) in all of these aspects and considered how this intersects with a wider adaptive radiation process.

In **Chapter 2,** I quantified the mandible shape of an F2 hybrid population and reported sexual dimorphism in mandible shape that was potentially ecologically relevant. I then used this data in combination with genotype and population genomic data and reported a new candidate gene for mandible shape, *zeb1*. In **Chapter 3,** I investigated differences in mandible shape between species and sexes in the two parental species for the F2 cross and reported sexual dimorphism in both *Tropheops* "Red Cheek" (TRC) and *Labeotropheus fuelleborni* (LF). I then used two finite element models from each of the parental species for each sex and two from the F2 population to investigate how the mandible reacts to loading that could be experienced during foraging. I reported evidence of sexual dimorphism in stress response in both species with males able to cope with greater loading on the mandible than females. Furthermore, I discussed unique structural adaptations in LF with the "wings" and in TRC, the "ridge", which seem to be adaptations to their respective feeding modes. In the hybrids, there was asymmetry in the loading pattern in the males and both sexes contained aspects of variation relating to the parental phenotypes. This finding suggests that there could be a wide range of stress responses in hybrid populations that provide functional consequences. In **Chapter 4**, I conducted a diet treatment experiment to investigate the possibility of sexual dimorphism in plasticity. Specifically, I measured the

plasticity of craniofacial shape and three functionally relevant traits. I found no evidence of sexual dimorphism in plastic response for either set of traits suggesting that plasticity is not required to maintain divergence between the sexes. In this final discussion chapter, I will discuss how the results reported in this thesis relate to existing literature and their contributions to the field of evo-devo in general. In addition, I will discuss limitations of the studies conducted in this thesis as well as some suggestions for future directions for research.

5.2 Exploring Mechanisms of Adaptive Divergence

5.2.1 Adaptive Radiations

Adaptive radiation is an evolutionary process whereby multiple species diverge from a common ancestor to allow them to exploit different ecological niches (Schluter, 1996). As these adaptations often result in morphological divergence and lead to a wide variation of phenotypes, adaptive radiations lend themselves to research focused on understanding the evolution of complex, morphological traits. There are numerous examples of adaptive radiation where divergence in morphology has been studied, some examples which have been discussed in detail throughout this thesis include Darwin's finches of the Galapagos islands, the *Anolis* lizards of the Caribbean and the Cichlids of the African Great Lakes (Fryer and Iles, 1972; Grant, 1986; Schluter, 1996, 2000; Losos, 1998). As well as studying the link between form and ecology, understanding the functional basis of adaptive traits is a key part of understanding the evolutionary process of divergence (Losos, 1990). In the *Anolis* lizards, a difference in limb length amongst ecomorphs translates to a functional trade-off between being able to move fast along wide perches, or move carefully and efficiently along narrow perches and thus divergence in how they navigate their environment (Irschick, 2002). Furthermore, because of the wide variation in phenotypes, adaptive radiations are also useful systems for exploring the genetic basis of important morphological traits such as the craniofacial skeleton. Conceptually, the field of evo-devo is now beginning to move towards a more integrative approach for

studying the evolution of morphological traits by exploring genes, function and morphology and how they link together (Irschick *et al*. 2013).

As well exploring the evolution of morphological traits, understanding variation within an adaptive radiation can also shed light on the process itself. In addition to the major axis of adaptive divergence in a radiation, for example the biting and suctioning axis of divergence present in numerous fish taxa (Albertson *et al*. 2005), there can also be smaller scale divergence that can contribute to the overall process adaptive radiation. Such divergence is termed "nested variation" and can result in small, but ultimately important phenotypic differences (Foster, Scott and Cresko, 1998; Parsons *et al*. 2015). By studying potential examples of nested variation, not only can we increase our understanding of the process of adaptive divergence and adaptive radiation, but we can also discover more about the evolution of complex morphological traits.

5.2.2 Ecological Sexual Dimorphism

In addition to the widely studied interspecific level, ecological divergence can exist between the sexes, otherwise known as ESD (Shine, 1989). A cause for ecological divergence between the sexes can be difficult to confirm because sexual dimorphism can also be the result of sexual selection, sexual size dimorphism or differences in reproduction or parental care between the sexes (Bolnick and Doebeli, 2003). The hypothesis of ESD is more likely when the trophic morphology differs between the sexes but is not undergoing sexual selection and the ecological divergence is not due to differences in body size as this would indicate niche divergence (Shine, 1989; Bolnick and Doebeli, 2003). ESD as a type of adaptive divergence has received limited attention although some notable examples in two key adaptive radiations, the *Anolis* lizards and threespine sticklebacks suggest that ESD could be widespread in nature (Butler and Losos, 2002; Cooper, Gilman and Boughman, 2011). Furthermore, sex differences are rarely taken into account in studies on adaptive divergence or radiation. The contribution of ESD to the wider process of adaptive radiation is unknown, but it could be a type of nested variation; indeed, adaptive radiation

could be a culmination of different levels of nested ecological divergence between species and sexes (Parsons *et al*. 2015).

5.2.3 Phenotypic Plasticity

Phenotypic plasticity, the ability of a genotype to produce multiple phenotypes in response to environmental conditions, is believed to be a generator of variation that leads to adaptive divergence (Pfennig *et al*. 2010). Furthermore, phenotypic plasticity could have a key role in the process of adaptive radiation; the 'flexible stem' model proposes that plasticity in the ancestral population, which ultimately reflects the pattern of divergence exhibited by the radiation, helps to facilitate the radiation process (Wund *et al*. 2008; Pfennig *et al*. 2010). Yet to be considered is the impact of plasticity on other mechanisms of adaptive divergence such as sexual dimorphism. If males and females differ in their plastic response, and therefore their ability to react to changing environmental conditions, this could contribute to adaptive phenotypic divergence between the sexes. Sexual dimorphism results from a difference in selection pressures (Cox and Calsbeek, 2009). Dimorphism between the sexes could be due to a combination of factors including ESD, sexual selection and parental care (e.g. Shine, 1989; Bolnick and Doebeli, 2003). If males and females are ecologically divergent but the phenotype of one sex is also constrained by another factor, a reduction in plasticity for that sex could alleviate any conflict that could arise from having flexibility in the phenotype. For example, in some African cichlids, females are mouthbrooders and consequently have a reduced bite force compared to males (tkint *et al*. 2012). Therefore, a reduction in plastic response could enable female cichlids to retain a phenotype which allows them to carry eggs efficiently whilst also reducing competition for resources with males.

5.3 Contributions to the Field and Limitations to Consider

By using multiple methods, including morphometrics, genetics and functional work, this thesis has adopted an integrative approach to answer evolutionary questions about adaptive divergence. Understanding the genetic basis of divergent traits is not only key to understanding how a complex morphological trait evolves, but also how the process of adaptive radiation itself proceeds (Irschick *et al*. 2013; Parsons and Albertson, 2013). In **Chapter 2**, the genetic basis of the mandible was explored using 3D morphometrics, quantitative trait loci (QTL) mapping and population genomic data. The work conducted in **Chapter 2** complements the existing knowledge the genetic basis of the mandible has been studied using 3D morphometrics and multivariate QTL mapping in African cichlids. Although there have been previous studies of the mandible in cichlids using a QTL approach (Albertson *et al*. 2005; Parsons and Albertson, 2009; Parsons, Marquez and Albertson, 2012), By studying fine-scale phenotypic divergence between two species that share a similar ecology, this work has highlighted a new candidate gene, *zeb1*, for cichlid craniofacial studies. Furthermore, I reported sexual dimorphism in the mandible shape and QTL models that differed depending on whether sex was included as a covariate or not; this suggests that sex has had a role in the evolution of the cichlid mandible. These results can be applicable to other systems as craniofacial development is a conserved process amongst vertebrates (Powder and Albertson, 2016); further work would be needed in other systems to complement this and to further elucidate the role of *zeb1* in the evolution of the mandible.

To further substantiate *zeb1* as candidate gene, comparison of gene expression between species and sexes would be of interest. Furthermore, there was no evidence of clear gene expression in the mandible in the TRC or LF samples but rather expression was throughout the craniofacial region. This does not mean that *zeb1* is not a reliable candidate, but that further work in more developmental stages is required. Given that *zeb1* has been implicated with neural crest cells (NCCs) which are present early in development then earlier developmental stages, such as between days 2 and 4 when the head is first

appearing, should be investigated. With regards to sex, a limitation is that expression *zeb1* would not be able to be investigated until sexual maturity due to the ambiguity around sex determination in cichlids (discussed in **Appendix 1**). Despite the general acceptance that these cichlids possess a sex determination system on LG7, at present there is no molecular approach for determining sex in Lake Malawi cichlids (Ser, Roberts and Kocher, 2010). In *Astatotilapia calliptera*, another Lake Malawi cichlid, the link between sex and genotype at various markers on LG7 was strongest at the marker nearest the gene *gsdf* which is responsible for sex-determination in other fishes (Peterson *et al*. 2017). Indeed, one marker in particular matched completely with sex in two Lake Malawi cichlids *A. calliptera* and *Metriaclima mbenjii* and could be an important candidate for sex determination in LF and TRC.

To answer questions relating to functional divergence between species and sexes, finite element analysis was conducted in **Chapter 3**. A technique growing in popularity in the field of evo-devo, this can be used to assess functional and structural adaptations in response to loading scenarios (Rayfield, 2007). Rather than looking at biting force transmitted from muscles through the bone, this chapter was a comparative analysis of how the mandible of different species and sexes cope with loading that would be expected during foraging. Both LF and TRC showed differences in stress patterns across the mandible as expected and the results reported in this chapter identify some morphological adaptations to handling loading. As LF scrapes and bites algae off of rocks it is reasonable to suggest they utilise the full width of the jaw when feeding as this could allow them to capture more food. Notably, presence of "wings" on the mandible which probably allows them to distribute the loading across an increased mandible width (see Figures in **Chapter 3**). On the other hand, TRC plucks algae and has a relatively narrower mandible in comparison as it likely only uses a small portion of the width of the mandible to feed on the strands. In **Chapter 3**, both TRC models showed minimal stress across the midline of the mandible compared to LF when loading was placed on a narrow width of the mandible. As well as differences in shape and function, the two species also differed in their plastic response with LF possessing a slightly more robust phenotype than TRC after the diet treatment experiment in **Chapter 4**; although there was more

plasticity in LF craniofacial shape than expected (Parsons *et al*. 2014). LF possesses a highly specialised phenotype, and their mandible morphology is unique in comparison to other species such as TRC and *Maylandia zebra* (Albertson and Kocher, 2001). The ability to dissipate stress when scraping has perhaps come at the expensive of having a more flexible phenotype. The results from **Chapter 3** contribute to our understanding of adaptive divergence and suggests that small-scale variation between species can be an important component of the radiation process.

There are a few limitations to consider for the FEA work conducted in **Chapter 3**. For FEA to be biologically relevant, accurate material properties should be applied and the final results validated (Panagiotopoulou, 2009). Material properties describe the elasticity of the bone and ideally should be experimentally derived (Peterson and Müller, 2018). However, whether accurate material properties are required or not is dependent on the question and using material properties from a similar species is acceptable (Peterson and Müller, 2018). The material properties applied in **Chapter 3** were based on experimentally derived results in tilapia (*Oreochromis aureus*) (Cohen *et al*. 2012) and they were kept consistent across samples because previous work has shown little difference in bone density between cichlids (Albertson, Cooper and Mann, 2012). Research has shown that altering the material properties may affect the quantitative stress and strain values but the distribution of stress on the model is the same (Strait *et al*. 2005). Therefore, when the goal is a comparative, structural study, as in **Chapter 3**, holding the material properties constant across samples is acceptable. To properly validate the results of FEA, the data should be compared to *in vivo* stress and strain data (Dumont, Piccirillo and Grosse, 2005). However, this can often be challenging in practice as it can involve placing a strain gauge on the bone (Richmond *et al*. 2005); this would be incredibly difficult to do in cichlids as the smallest commercially available strain gauges (approximately 0.3 x 1.96mm^2) are too large to fit on a cichlid face without interrupting natural feeding behaviour. Nonetheless, as the work presented from the FEA in **Chapter 3** is a comparison of the structure and ability of the mandible to cope with loading, these limitations are not major.

The possibility of ecological divergence between the sexes has received limited attention within the context of adaptive divergence yet the evidence presented in this thesis suggests it should be a more important consideration in evolutionary studies. Sexual dimorphism in mandible shape was reported in **Chapters 2** and **3** and in **Chapter 3** the finite element analysis (FEA) showed that male TRC and LF mandibles showed reduced levels of stress in response to loading in comparison with the females. This aligns with the idea of male cichlids having more of a "biting" phenotype than females (tkint *et al*. 2012; Parsons *et al*. 2015; McWhinnie and Parsons, 2019). The results from **Chapter 3** show a functional consequence of divergent phenotypes between the sexes and suggest this is ecologically relevant and is likely a case of ESD. As a concept, ESD is difficult to show unambiguously and this is especially true in cichlids. As discussed throughout, female cichlids are mouthbrooders and males use their mouths for fighting; as a result, females would benefit from having less musculature in the head region to add more space in to hold eggs, but conversely males would benefit from being able to be better biters for these aggressive interactions (tkint *et al*. 2012). As a result of these different selection pressures and potential ESD between the sexes, the prediction in **Chapter 4** was that females would show a reduction in plasticity in comparison to males after a diet treatment experiment. My results contradicted this prediction and showed there was no difference in plasticity between the sexes. This suggests that phenotypic plasticity does not contribute to divergence between sexes and that sexual dimorphism in mandible and craniofacial shape is under genetic control.

With this in mind, it is difficult to establish whether ecological divergence between the sexes in cichlids has evolved secondarily to differing selection pressures. It is currently unknown how sexual dimorphism contributes to the process of adaptive radiation with evidence of ecological differences between the sexes in *Anolis* lizards (Butler, Sawyer and Losos, 2007), and threespine sticklebacks (McGee and Wainwright, 2013). The evidence of ecologically relevant differences in shape and function between the sexes presented in this thesis show that sexual dimorphism could play a key functional role in radiation and show the importance of considering sexual dimorphism in an evolutionary context.

5.4 Understanding Mechanisms of Adaptive Divergence

By exploring mechanisms of adaptive divergence, we can enhance our understanding of how complex morphological traits evolve. Adaptive radiations are excellent models with which to study this as they are characterised by a diverse range of phenotypic variation (Schluter, 2000). By investigating the genetic architecture of these complex traits, this can provide an understanding of how the process of adaptive radiation arises (Irschick *et al*. 2013). The three-stage model of adaptive radiation posits that vertebrate radiations follow a generalised trend of divergence in habitat, trophic morphology and then sexual selection (Streelman and Danley, 2003). Therefore, as the process is posited to be broadly similar in all taxa, findings from studying divergence in the African cichlid radiations can be applicable to other key radiations. Currently it is unknown exactly how this process proceeds, but the work conducted in this thesis suggests divergence between sexes could form part of the divergence in trophic morphology stage. Indeed, adaptive radiations could be comprised of multiple layers of fine-scale divergence (Parsons *et al*. 2015), therefore exploring sexual dimorphism in tandem with exploring interspecific adaptive divergence could be important to broaden our understanding of this process.

Notably, radiations exhibit a wide range of phenotypic variation and we can enhance our understanding of how complex traits, such as the craniofacial skeleton, evolve by studying adaptive divergence within a radiation. The craniofacial skeleton represents a primary point of contact with the environment and determines what food can be eaten and how efficiently it can be consumed; the mandible in particular is a key innovation in vertebrates (Parsons and Albertson, 2009). Despite this importance to evolution and adaptation, the underlying genetic architecture of craniofacial traits is relatively unknown due the complexity of the variation (Roberts *et al*. 2011; Irschick *et al*. 2013). An insightful way to explore the evolution of a morphological trait, such as the craniofacial skeleton, is to compare candidate gene expression across species which exhibit divergence in morphology but share a common genetic background as in cichlids (Parsons and Albertson, 2009). Furthermore, development of the craniofacial skeleton is conserved across vertebrates meaning that findings from

cichlids (and other radiations) could be relevant and applicable to other vertebrate taxa (Powder and Albertson, 2016).

As well as enhancing our understanding of the evolutionary process, understanding mechanisms of adaptive divergence has broader implications for human health and disease. African cichlids in particular exhibit a wide range of craniofacial phenotypes which represent both normal and clinical craniofacial variation in humans (Albertson *et al*. 2009; Powder and Albertson, 2016). By exploring adaptive divergence of the craniofacial skeleton, particularly the mandible, this can help elucidate more candidate genes of relevance to human craniofacial disorders (Albertson *et al*. 2009).

5.5 Future Research Directions

The mandible is a key vertebrate innovation and whilst there are a number of candidate genes for the development of the mandible, there are still many avenues to explore. Future research should look towards exploring the newer candidate genes (including *zeb1*) in more depth using small molecule experiments, where the normal development of the mandible is perturbed by a chemical agonist/antagonist (Parsons *et al*. 2014), and perhaps *qPCR* to quantify expression levels in different species. This thesis has looked at small scale adaptive divergence between TRC and LF, but this work could be expanded to include another species on the other end of the Lake Malawi feeding dichotomy, *Maylandia zebra* (a suction-feeding generalist) to comparatively explore *zeb1* further. By expanding and looking at additional species, this would help us further understand how the mandible and craniofacial morphology has diversified (Irschick *et al*. 2013).

With regards to sexual dimorphism, while I show sexual dimorphism in mandible shape in TRC and LF, and there are multiple examples of sexual dimorphism in African cichlids in the literature as discussed throughout this thesis (e.g. tkint *et al*. (2012); Parsons *et al*. (2015); McWhinnie and Parsons, (2019)), it would be interesting to investigate whether this dimorphism is widespread across Lake

Malawi cichlids and if a similar pattern is observed in the major adaptive radiations of Lake Victoria and Lake Tanganyika. This thesis has only considered sexual dimorphism in two species but broadening this investigation to assess whether or not some species are more sexually dimorphic than others would be an interesting avenue to explore.

Although there was no evidence of sexual dimorphism in plasticity, an experiment leading on from the work conducted in **Chapter 4** could elucidate what effect plasticity has on mouthbrooding in female cichlids. I would expect that mouthbrooding would be negatively affected by plasticity; such an experiment could assess the number of eggs a female can hold at a time and compare this with fish which have been under a different diet treatment. It could be that high levels of plasticity in females leads to trade-offs in mouthbrooding ability. It could also be that other aspects of the craniofacial region not examined in **Chapter 4**, such as the ventral view of the fish, do differ in plasticity between sexes. The ventral view can be used as a way to infer width of the mandible and the part of the buccal cavity of the fish, where eggs are held. A hypothesis would be that females are able to respond to fluctuating environmental conditions in the same way as males by having a reduction in plasticity in the ventral view (and therefore, the buccal cavity). This could act to reduce the potential negative impacts of plasticity on the ability to mouth-brood, if any exist.

5.6 Conclusions

A central focus for the field of evo-devo is understanding the mechanisms underlying adaptive phenotypes. With a wide range of craniofacial variation that has evolved over a relatively short time frame, African cichlids from Lake Malawi are an excellent system with which to test and explore this. I have utilised an integrative approach to investigate evolution of the mandible and craniofacial skeleton and considered adaptive divergence between both species and sex. By mapping the relationship between genotype and phenotype, this work has identified a new candidate gene in the genetic architecture of the mandible,

zeb1. Despite sharing a similar ecology, TRC and LF differ in shape and how their mandible reacts to applied loading probably experienced during feeding. Sexual dimorphism in mandible shape was present in the hybrid cross and both parental species and alongside the results from the FEA this suggests male and female phenotypes are divergent in an ecologically relevant way. However, there was no difference in plastic response between the sexes suggesting phenotypic plasticity does not play a role in maintaining divergence between the sexes. As male and female cichlids face differing pressures on their craniofacial skeleton relating to reproduction, it is challenging to disentangle whether ecological divergence between the sexes has evolved as consequence of this. Nonetheless, sexual dimorphism has likely played an important role in the evolution of the cichlid craniofacial skeleton, and specifically the mandible. Taken together, the work presented in this thesis shows that small-scale divergence between species and sexes can be important both functionally and ecologically and could explain the success of adaptive radiations.

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Appendix 1: Sex Determination: A Genomic Puzzle in Cichlids

Note: This is my contribution to the book chapter "An evo-devo view of post-genomic African cichlid biology: enhanced models for evolution and biomedicine" in collaboration with K.J. Parsons and T.A. Armstrong which has been submitted for review and publication.

The topic of sex determination has captured the attention of evolutionary biologists for decades. African cichlid genomics is now quickly enhancing our understanding of the variety of sex determination systems present in nature. Cichlids challenge the main consensus for sex determination which involves separate sex chromosomes (Charlesworth 1991, Hodgkin 1992). Likewise, it can be argued from a range of research that sex determination is not as clear cut in humans as was once thought. For example, differences of sex development (DSDs) in humans are relatively common with a one in 5,500 incidence rate, with clear chromosomal abnormalities being involved in some instances but also range of genetic and environmental mechanisms contributing in others (Kousta, Papathanasiou and Skordis, 2010). Thus, the range of mechanisms for sex, as well as sex-linked traits in cichlids provide potential clinical, as well as evolutionary relevance.

Under the broadest conventional mechanism sex chromosomes are structurally different with only minimal opportunity for genetic recombination between them. Most commonly male heterogameity exists; whereby males possess the heterozygous chromosomal combination XY and reduced levels of genetic recombination whereas females are XX and have increased levels of recombination. A possible but less common situation is female heterogameity whereby, females are heterozygous with the chromosomal combination ZW, but males are homozygous ZZ. The above conventions are assumed to be the most common, since the genetics of sex determination has focused on mammals and model organisms (Batchtrog *et al*. 2014). However, several other mechanisms for sex determination exist across taxa with fish (including cichlids) showing a relatively large range of mechanisms. For example, an individual's sex can also be determined by environmental mechanisms; factors including pH and

temperature determine sex in Apistogramma cichlids (Römer and Beisenherz, 1996). Sex determination can also be dependent upon a complex of genes (i.e. polygenic sex determination). So far complex polygenic systems for sex determination have been found in widely different species of fishes. In polygenic sex determination, the genes involved are likely to be spaced across the genome (Liew *et al*. 2012), while providing more targets for selection this also increases the probability that they are subject to environmental influences. Polygenic mechanisms for sex determination have been documented in European seabass (*Dicentrarchus labrax*) where a factorial mating study carried out by Vandeputte et al. (2007) showed evidence of a polygenic system where there is both a genetic and environmental component. Similarly, in zebrafish (*Danio rerio*) the exact workings of the sex determination mechanism is relatively unknown; however, a recent study by Liew *et al*. (2012) suggests a polygenic system which is primarily genetic with a small input from environmental factors. It is clear that perhaps most challenging for future research will be cases whereby complex interactions between genetic and environmental factors determine sex. These uncommon mechanisms of sex determination remain a largely open set of questions for genomic approaches to address in cichlids.

But why do fish exhibit a more diverse range of sex-determining mechanisms than other groups (e.g. mammals)? This question is largely unanswered, but perhaps the range of mechanisms present within cichlids will provide more comparative power than more distantly related groups. At a basic level, chromosome cytology studies show that sex chromosomes themselves are relatively more difficult to distinguish in fishes than in mammals, suggesting perhaps that recombination is more likely. For African cichlids, there are a wide variety of mechanisms of sex determination which can vary between species and in the case of Lake Malawi, have evolved fairly recently in the timeline of the radiation (Ser, Roberts and Kocher, 2010). Recent findings from seven species of Malawi cichlids suggest that 'B chromosomes', revealed by genomic sequencing, are only present in females and thus could be important for determining sex (Clark et al. 2016). Similarly in Lake Victoria, twelve species are known to possess 'B' chromosomes in addition to the standard 'A' chromosomes that function in sex determination (Yoshida *et al*. 2011; Kuroiwa *et al*. 2014). A

single B chromosome provides two potential mechanisms for their maintenance in these populations. These driving mechanisms are biased toward females in other plants and animals with B chromosomes and include nondisjunction or preferential segregation in a mitotic division prior to the germ-line, or another mechanism whereby preferential segregation takes place during meiosis I. Currently, preferential segregation during meiosis 1 appears to be the favoured hypothesis (Kuroiwa *et al*. 2014) but a better understanding of what causes B chromosomes to be exclusively present in females is needed to appreciate their evolutionary impacts.

For African cichlids, environmental sex determination has been documented in both Nile Tilapia (*Orechromis niloticus*) and the orange chromide (*Etroplus maculatus*) (Barlow 2008). The orange chromide example is noteworthy because this species is basal to extant lineages of cichlids which suggests that perhaps environmental sex determination is an ancestral condition. More specifically, the sex determination system of the Nile Tilapia is believed to be predominated by male heterogameity with a major sex-determining locus. In addition, it appears that other loci are involved because not all families display evidence of sex linkage to this marker (Lee, Penman and Kocher, 2003). There is also evidence that temperature and social conditions can have an effect on sex determination in the cichlids. In Nile Tilapia, sex determination is not exclusively controlled by genetic factors (Barlow, 2008). Work by Baroiller *et al*. (1995) demonstrated that high temperatures can overrule genetic factors and that sex determination in this species is a combination of environment, sex chromosomes and interactions between the two. To date, environmental inputs have surprisingly been largely ignored in studies of sex determination focused on African cichlids from the Rift Lakes. We suggest that incorporating the G-P-E view may aid researchers currently undertaking the difficult task of uncovering the mechanisms of sex determination at a genomic level.

Currently, studies more specific to Rift lake cichlids have focused on major sex determining systems. For example, data from Ser, Roberts and Kocher (2010) show that 19 species of Malawi *Maylandia* cichlids exhibit both male and female heterogameity across species. During this study, single pair lab-based crosses

were made for each species to allow a detailed investigation of the sex determination systems present. Notably, for the species *Metriaclima pyrsonotus*, Ser, Roberts and Kocher (2010) reported that both systems were found to segregate within a single family. In this case, the ZW system is epistatically dominant to the XY system when both of the dominant loci involved in sex determination (Z and W) are present within an individual. Additionally, a number of families of several species showed no linkage of sex to markers to their usual locations on linkage groups 7 (associated with male heterogameity) or 5 (associated with female heterogameity), which suggests that any number of genes on different chromosomes could be controlling sex determination (Ser, Roberts and Kocher, 2010). Further genetic mapping studies have confirmed such additional mechanisms for sex determination in Lake Malawi cichlids as a study on two mbuna species by Parnell and Streelman (2013) reported the ZW locus on LG5, two XY loci on LG 7 and two additional loci detected on LG 3 and LG 20 which interact with these and influence sex determination.

What would cause such a range of sex determining mechanisms to evolve in cichlids? It has commonly been suggested sex determination could evolve to resolve sexual conflicts. Such resolutions are favoured because of sexually antagonistic selection whereby a trait that is beneficial to one sex can have a detrimental effect on the other leading to genetic conflict (van Doorn and Kirkpatrick, 2007; Bachtrog *et al*. 2014). To investigate genetic conflict in Malawi cichlids, Roberts, Ser and Kocher (2009) examined the 'orange blotch' (OB) colouration that is present in 20 species. The OB phenotype is found almost entirely in females and is considered a method of camouflage by disruptive colouration; conversely this phenotype has a negative effect on males that rely on bright colouration to attract mates. The gene associated with the OB phenotype is *Pax7* and as there are no differences in the coding sequence of *Pax7*, Roberts, Ser and Kocher (2009) suggest that the OB phenotype is the result of cis-regulatory differences in *Pax7*. To resolve sexual conflict, it was suggested that the OB (*Pax7*) locus is tightly linked to a dominant female sex determiner (W). The OB-linked ZW sex determination found on LG5 is epistatically dominant to the male heterogametic system found on LG7, which is understood to be the ancestral condition for sex determination in Lake Malawi cichlids (Roberts, Ser

and Kocher, 2009; Ser, Roberts and Kocher, 2010). The linkage between the OB phenotype and ZW locus has been confirmed by Parnell and Streelman (2013).

In the case of the OB phenotype, there are two potential paths to the resolution of the sexual conflict. First, the sexually antagonistic selection needed at the OB locus could have allowed for the appearance and linkage with a female sex determiner (W) nearby on LG5 to provide a resolution. Or, the OB polymorphism may have emerged near a newly evolving sex determiner which then allowed for the resulting OB-W linkage to evolve and exist alongside the original ancestral system (XY) (Roberts, Ser and Kocher, 2009). However, it is also possible that the OB phenotype has evolved subsequently to, rather than driving the evolution of the ZW mechanism (van Doorn and Kirkpatrick, 2007). The OB phenotype and its linkage with sex determination has provided a solution to sexual conflict of this sexually antagonist trait.

Linkage with sex determination could be also be the case for a variety of other traits in African cichlids (Roberts, Ser and Kocher, 2009). Work by Parsons et al. (2015) on a F2 hybrid cross of *Labeotropheus fuelleborni* and *Tropheops* "Red Cheek" (Lake Malawi cichlids) found evidence of sexual shape dimorphism in the craniofacial region potentially related to ecologically relevant differences between the sexes; males of this hybrid cross had a steeper craniofacial profile compared to a gradual, sloping profile in females. Furthermore, QTL analyses performed suggested that loci involved with craniofacial shape are often linked to sex determining loci on the same linkage group or are epistatically influenced by sex. For example, the QTL for the discriminant function scores (the axis of sex in this study) was located on LG7, the same linkage group as the sex determining loci. At this QTL, the *T.* "Red Cheek" allele (male in the original parental cross) resulted in a steep 'male-like' craniofacial profile whereas the *L. fuelleborni* allele (female in the parental cross) resulted in a more sloping 'female-like' profile. Notably, it has also been suggested that QTL for morphological plasticity are also linked to sex determining loci on LG 7 (Parsons *et al.* 2016). Therefore, cichlids may utilize a number of interactions between sex and morphology to resolve genetic conflict between the sexes. Although further details have yet to emerge genetic conflict may bias the direction of

adaptive divergence as patterns of sexual dimorphism align with the major trajectory of morphological divergence in Malawi (Parsons *et al*. 2015). Avoiding such biases could explain why so many mechanisms for sex determination persist in African cichlids, and potentially explains why speciation events in African cichlids have been far more numerous than in other adaptive radiations.

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Appendix 2: Conference Abstracts

Talk at 2nd Biennial Meeting Pan-American Society for Evolutionary Developmental Biology, August 2017, University of Calgary

Note: This abstract was also submitted and presented as a poster at the UK Evo Devo Symposium, September 2017, Natural History Museum London.

Understanding connections between adaptive phenotypes and the mechanisms underlying them provides a central focus for evo-devo. The changes in these connections can occur through adaptive divergence, a phenomenon usually studied between species. Ecological sexual dimorphism (ESD) represents another form of adaptive divergence that evolves between sexes due to alternate ecological conditions and can result in differences in trophic morphology, a key feature of many adaptive radiations. Here, I explore adaptive variation in trophic morphology using the African cichlid mandible. Sexual dimorphism in colour and body size is prevalent amongst cichlids, suggesting sexual selection, but potential adaptive differences between sexes are rarely considered. Therefore, I combine techniques from evo-devo and engineering to test for evidence of ESD. In this project, I use 3D measures of shape and QTL mapping to determine the genetic basis of differences between species and sexes. In addition, as it has been well established that phenotypic plasticity is itself an evolvable trait, I examine whether developmentally plastic responses to alternate foraging environments are influenced by sex, and test whether these sex-specific developmental responses are adaptive. Taken together, this research addresses ESD from a developmental and genetic perspective to provide a wider understanding of how adaptive divergence proceeds.

Poster at 7th Meeting of the European Society for Evolutionary Developmental Biology (EED) June 2018 at National University of Ireland

Note: This poster won a prize and was scored in the top 10 for submissions at the conference.

Understanding connections between adaptive phenotypes and the mechanisms underlying them provides a central focus for evo-devo. Changes in these connections can occur through adaptive divergence, a phenomenon usually studied among species. Ecological sexual dimorphism (ESD) represents another form of adaptive divergence that evolves between sexes due to alternate ecological conditions and can result in differences in trophic morphology, a key feature of many adaptive radiations. Here, I explore adaptive variation in trophic morphology using the genotype to phenotype relationship of an African cichlid mandible. Sexual dimorphism in colour and body size is prevalent amongst cichlids, suggesting sexual selection, but potential adaptive differences between sexes are rarely considered. Therefore, I combine techniques from evo-devo and engineering to examine shape and biomechanical function to test for evidence of ESD. Further, I use 3D measures of shape of the mandible combined with multivariate quantitative trait loci mapping (QTL) to determine the genetic basis of differences between species and sexes. These results are complemented by population genomic data to provide candidate genes for functional investigations including gene expression assays and small molecule experiments during key periods of jaw development. Taken together, this research addresses ESD from a developmental and genetic perspective to provide a wider understanding of how adaptive divergence proceeds.